UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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A todos os animais que participaram neste estudo, e a todos aqueles que se cruzarão na minha futura vida profissional e que espero possam beneficiar da investigação que se iniciou com este trabalho.

AGRADECIMENTOS:

E como nenhum percurso é feito sozinho, gostava de deixar aqui um agradecimento a todos os que me acompanharam durante esta enorme viagem que foi o curso de Medicina Veterinária na FMV.

Um enorme obrigado à Professora Constança Pomba que me recebeu desde o primeiro ano na faculdade e me abriu as portas para o mundo da microbiologia clínica e da investigação, que me acompanhou durante todos os anos deste curso e me deu, não só conhecimento, mas também apoio, força e motivação para seguir e pensar sempre mais alto. É um grande exemplo para mim e espero poder continuar a trabalhar e a contribuir para o seu vasto trabalho científico.
Ao Doutor Rodolfo Oliveira Leal, que começou como meu coorientador, mas que terminou como uma inspiração para mim e para todo o percurso que desejo seguir no mundo da Medicina Veterinária. Quero agradecer por tudo aquilo que me ensinou e por ter feito a minha paixão por Medicina Interna escalar, mas também por todos os momentos de boa disposição, confidência e amizade que existiram. Espero poder continuar a contar com o seu apoio e partilha científica.
À Engenheira Adriana Belas, que me recebeu, iniciou no trabalho de laboratório e que foi uma grande ajuda neste intenso trabalho. Foram muitas as horas sentadas na bancada lado a lado, mas também muitas as horas de amizade e partilha. Obrigada pelo apoio e ensinamentos.
Um agradecimento ao Professor Doutor João Carriço, Dra. Catarina Inês Mendes do Instituto de Medicina Molecular e Dra. Belen Carbonetto do Instituto Gulbenkian de Ciência que me ajudaram na análise bioinformática constante neste trabalho.
A toda a equipa do Laboratório de Resistências aos Antimicrobianos e Biocidas que proporcionaram um excelente ambiente de trabalho. À Dra. Cátia Marques, que nunca se cansou de me ensinar e ajudar a melhorar, à Juliana que veio trazer um pouco de equilíbrio com a sua calma exemplar e à Pâmela, a minha amiga carioca que transmite sempre boa disposição e uma palavra de apoio (e umas citologias interessantes claro).
A todos os médicos, enfermeiros, auxiliares, rececionistas e, claro, colegas estagiários do HEFMV, pelo conhecimento transmitido e pelo excelente ambiente de estágio. Fiz amizades que de certo não esquecerei.
Às minhas derradeiras parceiras de viagem desde o dia um: Caroline, Mer, Tina e Lenka, mas também à Bea que chegou mais tarde, mas se tornou um peça tão importante como todas as outras. Sem vocês, nada teria sido tão divertido, tão bom, tão memorável. Foram muitos os momentos que partilhámos, uns excelentes, uns bons e outros não tão bons, mas em todos eles vocês estavam lá, e isso diz tudo. Quando dizem que os amigos da faculdade são para a vida de certeza que estão a falar sobre nós. *Linduxas* para sempre!!
Às minhas amigas "de sempre": Guida, Ana, Ana Maria, Beatriz e Inês, porque antes de iniciar esta viagem tive de aqui chegar, e sem vocês isso não teria sido possível. Obrigada por me ouvirem falar de assuntos sobre os quais nada percebiam e por tirarem a minha cabeça da FMV sempre que eu precisava, obrigada por compreenderem que a distância nem sempre permitia estar com vocês em momentos importantes. Love you all!!
À minha família que sempre me apoiou e acompanhou de todas as formas.
Ao meu avô Patão que infelizmente não pode presenciar o seu maior desejo que era o dia da minha formatura. Foi uma das pessoas que mais me apoiou nos meus primeiros anos de curso e todos os dias me dava um abraço cheio de força e motivação.
E *Last but not Least*, aos meus Pais, os meus primeiros heróis, os meus exemplos tanto a nível humano como a nível profissional. Não estaria aqui hoje sem vocês, sem o vosso apoio, sem a oportunidade que me deram (desde pequena) de me apaixonar pela linda profissão que é Medicina Veterinária, e principalmente por me incentivarem e ensinarem a seguir o meu próprio caminho e não o de mais ninguém. Foram a minha primeira inspiração e serão sempre a minha inspiração mais importante.

"Gratitude is the memory of the heart."
-Jean-Baptiste Massieu

Abstract

Gut microbiome in healthy dogs and cats

Recent studies show that the gut microbiome contributes to the vital physiologic and immunologic processes and is influenced by external factors such as diet, environment, medical interventions, and disease states. In this study, we describe the gut microbiome of healthy dogs and cats, from households and shelters, contributing for a better understanding of the effect that environment can have on it.

The samples were collected between 2016 and 2017 and consisted of a household group (N=38, N=26 dogs and N=12 cats) and a shelter group (N=62, N=51 dogs and N=11 cats). DNA extraction was done directly from the faeces and the V4 region of the 16S rRNA gene was amplified, followed by sequencing using Illumina MiSeq. Raw sequences were treated using QIIME2 and Greengenes database was chosen for taxonomic classification alignment at 99% similarity. SAS statistical software was used, a p-value < 0.05 was considered.

The Principal Coordinate Analysis plot demonstrated that the feline and canine microbiomes were well separated, as well as the household dog samples and the shelter dog samples, meaning that there is a difference in the gut microbiome between these groups. The same conclusion was not observed for the cat samples. The phyla *Firmicutes* and *Bacteroidetes* were the predominant ones in both species. For the dog samples, there was no difference between the two groups in *Firmicutes* and *Bacteroidetes* phyla ($p>0.05$), but the phyla *Fusobacteria* and *Proteobacteria* were in higher percentages in the shelter group ($p<0.05$).

To our knowledge, this is the first study that describes and compares the gut microbiome composition of healthy household and shelter dogs and cats. This study demonstrates that the environment where animals are born and grow, as well as the amount of contact they have with humans, may have a great influence in their gut microbiome.

Keywords**:** Gut microbiome, Dog, Cat, Animal, Healthy, 16S rRNA

Resumo

Microbioma entérico em cães e gatos saudáveis

Estudos recentes demonstram que o microbioma gastrointestinal tem um papel essencial nos processos fisiológicos e imunológicos do hospedeiro e que é fortemente influenciado pela dieta, ambiente, intervenções médicas e estados de doenças. Neste estudo pretendemos descrever o microbioma entérico de cães e gatos saudáveis provenientes de canis e de casas, contribuindo para uma melhor compreensão do efeito que o ambiente pode ter neste.

As amostras foram colhidas entre 2016 e 2017 e consistiam num grupo de casa (N=38, N=26 cães e N=12 gatos) e um grupo de canil (N=62, N=51 cães e N=11 gatos). Foi realizada extração de DNA diretamente a partir das fezes e amplificada a região V4 do gene 16S rRNA, seguindo-se de sequenciação com Illumina MiSeq. As sequências foram tratadas usando o QIIME2 e a biblioteca Greengenes foi escolhida para classificação taxonómica, com o alinhamento a 99% de semelhança. Foi usado o SAS e considerado um *p*-value<0.05.

O *Principal Coordinate Analysis plot* demonstrou que os microbiomas felino e canino são diferentes, assim como o microbioma de cães de casa e canil. No entanto, não foi possível chegar à mesma conclusão nas amostras de gato. Os phyla *Firmicutes* e *Bacteroidetes* foram os predominantes em ambas as espécies sendo que, nas amostras de cão, não houve uma diferença estatisticamente significativa entre os dois grupos para estes phyla (p>0.05). Os phyla *Fusobacteria* e *Proteobacteria* foram identificados com maior frequência nas amostras de canil, tendo sido esta diferença estatisticamente significativa (p<0.05).

Tanto quanto é do nosso conhecimento, este é o primeiro estudo a descrever e comparar o microbioma gastrointestinal de cães e gatos saudáveis provenientes de casa e canil. Este estudo demonstra que o ambiente em que os animais nascem e habitam, assim como a quantidade de contacto que têm com o ser humano, pode ter grande influência no seu microbioma gastrointestinal.

Palavras-chave: Microbioma entérico, Cão, Gato, Animais, saudáveis, 16S rRNA

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Abbreviations

% - Percentage

< - Less than

= - Equal to

® – Registered trademark

µl – microliter

ANCOM – “Analysis of Composition of Microbiomes” , Análise de composição de microbiomas

DNA - Deoxyribonucleic acid

HP - High-protein diet

IBD - Inflammatory Bowel Disease

IGC - Instituto Gulbenkian da Ciência

MP - Moderate-protein diet

NGS - Next-generation Sequencing

nm - Nanometer

ºC – Centigrade degrees

OTUs - Operational Taxonomic Units

PCoA - Principal coordinate analysis

QIIME – Quantitative Insights Into Microbial Ecology

RNA – Ribonucleic Acid

rRNA - ribosomal Ribonucleic Acid

1 Internship Activities Report

The final internship of the Master Course in Veterinary Medicine was divided in two parts, 6 months in the Laboratory of Antimicrobials and Biocides Resistance (LRAB) of the Faculty of Veterinary Medicine – University of Lisbon, and 6 months at the Veterinary Teaching Hospital (HEFMV) of the same institution.

1.1.1 Laboratory of Antimicrobials and Biocides Resistance

The laboratory internship was performed under the supervision of Professor Maria Constança Pomba, starting from 1st September 2017 until 28th February 2018. In total I spent 1160 hours doing laboratory work.

During this time, I learned with Eng. Adriana Belas (MSc, Biotechonology Engineering) and Dr Catia Marques (MIMV MSc), the basis for laboratory work, from the security and asepsis rules to microbiology and molecular biology techniques. This knowledge gave me the opportunity to work autonomously in the laboratory and complete the tasks presented in this thesis, as well as to have contact with all the research projects that are undergoing in the LRAB.

Regarding the microbiology methods, were executed microbiological cultures, including the preparation of the culture mediums; Gram and Diff-Quick staining techniques; biochemical tests for bacteria identification; antimicrobials susceptibility tests (disk diffusion and minimum inhibitory concentration methods).

As for the molecular biology were learned and executed the following: Deoxyribonucleic acid (DNA) extraction and purification for gram positive and negative bacteria; polymerase chain reaction (PCR) technique for bacteria identification, resistance and virulence genes identification; and agarose gel electrophoresis.

Specifically, for my master thesis work, I learned with Eng. Adriana Belas (PhD student under supervison of Professora Constança Pomba) about gut microbiome studies, regarding effects of sample collection and storage, DNA extraction methods and bioinformatics analyses. All the methods learned and executed during the internship that were used for the present work are described in chapter 3.

1.1.2 Research communications

The work included in the master thesis study was submitted for CIISA 2018 Congress:

Belas A., Aboim, C., Leal, R., Mendes, C.I., Marques, C., Carriço, J.A., Pomba. C. (2018) Characterization of gut microbiome of healthy companion animals from households and shelters. [Oral communication] CIISA Congress 2018, 16-17 November 2018, Lisbon, Portugal. Submitted.

It is also predicted the writing of a scientific article regarding the work developed for the obtaining of the master's degree in veterinary medicine.

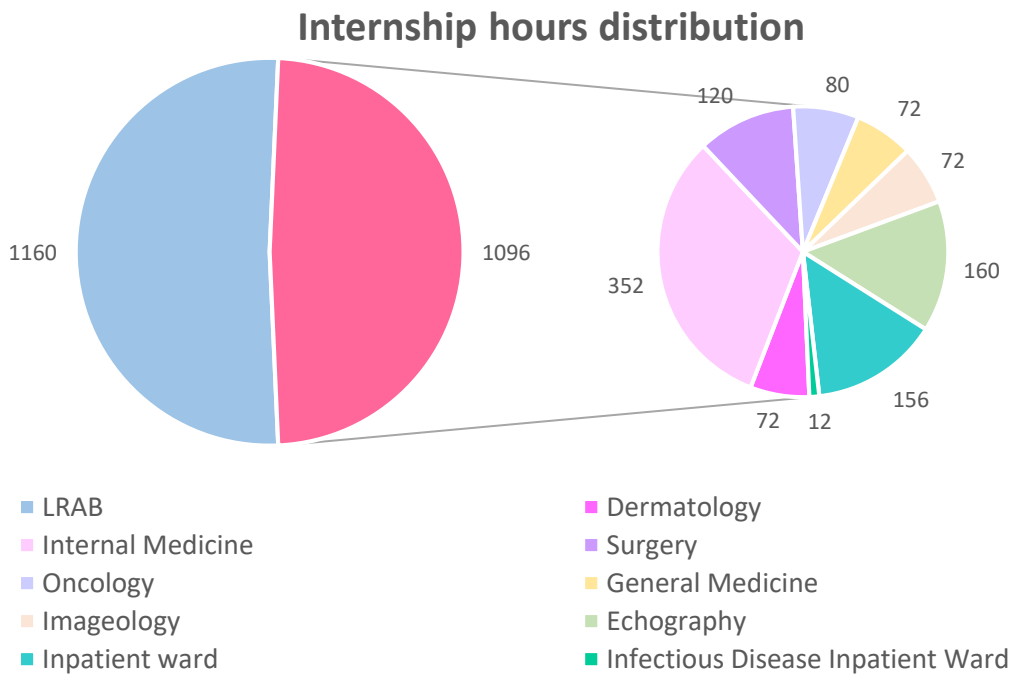
Since 2013, and simultaneously with the Veterinary Medicine Master degree, I worked as a volunteer at the Laboratory of Antimicrobial Resistance co-authoring the following scientific articles and research communications:

1.1.3 Teaching Veterinary Hospital

The second part of my internship was spent on the clinical rotations of the HEFMV under the supervision of Doctor Rodolfo Oliveira Leal, European Veterinary Specialist in Small Animal Medicine. The internship began in 1st March 2018 and ended in 31st August 2018, spending some total of 1096 hours in clinical rotations.

The shifts consisted of 8 hours (from 8 o'clock to 16 o'clock or from 13 o'clock to 21 o'clock) when working on the regular services or of 12 hours (from 8 o'clock to 20 o'clock or 20 o'clock to 8 o'clock) when working on the inpatient ward.

The clinical rotations included the following services: Internal Medicine (352 hours), Ultrasonography (160 hours), Inpatient Ward (156 hours), Surgery (120 hours), Oncology (80 hours), General Medicine (72 hours), Dermatology (72 hours), Imagiology (72 hours), and Infectious Diseases inpatient ward (12 hours).



Graph 1 - Internship hours distribution

The majority of my internship was spent in the Internal Medicine service with Doctor Rodolfo Oliveira Leal. On this service I had the opportunity to contact with referral cases appointments and learn about the recent developments in Internal Medicine, Endocrinology and Gastrointestinal cases were overrepresented. I was also challenged daily by my supervisor with medical discussions about differential diagnosis, complementary exams and therapeutic plans. I also had the opportunity to assist to several endoscopy procedures, mainly gastroduodenoscopies (with concurrent biopsy samples and foreign bodies removal) and colonoscopy (with concurrent biopsy samples), but also bronchoscopy and rhinoscopy (biopsies and foreign bodies removal).

During the Ultrasonography rotation, I had the opportunity to observe and execute several abdominal examinations, most of them related to gastrointestinal processes or urinary tract disease, but also reproductive tract. I also had the opportunity to observe a few thoracic ultrasounds for the diagnostics of cardiac abnormalities.

On the Inpatient Ward I had the responsibility to execute physical examinations to all the animals, as well as to administrate the medications and control the fluid therapy. It was also of my responsibility to do blood collections, blood and urinary catheterizations, cystocentesis and give the owners therapeutic and care advices at the time of the medical release.

Throughout Surgery rotation I had the responsibility to receive the patients, as well as collect all the clinical information about the patients and prepare them for surgery, including pre-medication, anaesthesia induction, tracheal intubation and anaesthesia. After the surgery I was in charge to monitor the patient until they were awake and to give the medical release. I also had the opportunity to participate as a surgeon's assistant in the following procedures:

spaying and castration; orthopaedic surgery, mainly hip dysplasia and bone fractures; neurological surgery, such as intervertebral disc hernia; oncological surgery, for the removal of nodules or intra-abdominal masses; and dentistry procedures, mainly comprehensive oral health assessment and treatment or simple.

The Oncology service included the participation in oncology appointments with the doctor responsible, as well as the chemotherapy sessions that occurred two times a week. During the chemotherapy sessions I had the chance to visualize and help with the preparation and administration of the chemotherapeutics.

During the General Medicine service, I was able to have contact with first advise appointments, emergency care, vaccinations, pre and post-surgery appointments, among others. On this rotation it was given me the responsibility to receive the animal as well as do the anamnesis, physical examination and discuss with the responsible doctor the differential diagnosis and possible approaches in terms of exams and therapeutics.

In the Dermatology service I had contact with numerous cases of dermatological diseases, mainly: atopic dogs, leishmaniosis, otitis and pyoderma. I was also able to do several complementary examinations such as skin swabs, skin scraping, cytology, otoscopies among others.

On the Imagiology department I had the chance to participate in the realization of X-rays and computerized tomography, including anaesthesia monitoring. I also had the chance to learn how to interpret the images and the characteristic images of several diseases.

2 Bibliographic Review – Gastrointestinal Microbiome

2.1 What is the Microbiome and its importance

Microbiome is the word used to identify the diverse consortium of bacteria, archaea, fungi, protozoa, viruses, and their collective genome that is present in the hosts body (Shreiner, Kao, & Young, 2015; Turnbaugh et al., 2007). Estimates have shown that the gut microbiota of animals consist of 10^{10}–10^{14} microbial cells, which is roughly similar or 10 times higher than the number of cells that belong to the host (Honneffer, Minamoto, & Suchodolski, 2014; J. Kim, An, Kim, Lee, & Cho, 2017; Sender, Fuchs, & Milo, 2016). Since the bacteria are the main constituents of the microbiome, this term is used in the majority the studies regarding only this domain.

The knowledge and research in this field was quite limited in veterinary medicine and little has been published until very recently. The advances in technology and the appearing of the next-generation sequencing (NGS) allowed a more thorough analysis and study of the gut microbiome (Kwong, McCallum, Sintchenko, & Howden, 2015).

Mammals, including dogs and cats, are metagenomic, meaning that they are constituted by both their own gene complements and those of the large community of microorganisms they harbour, the microbiome (Ley et al., 2008).

Recent studies have shown that microbiome makes crucial contributions in vital physiologic and immunologic processes including energy homeostasis and metabolism, endocrine signaling health of the gut epithelia, among others (Barko, McMichael, Swanson, & Williams, 2018; Nicholson et al., 2012; Shreiner, Kao, & Young, 2015; Turnbaugh et al., 2007). Microbiome may change due to external factors such as diet, environment, medical interventions, and disease states(Barko et al., 2018; Nicholson et al., 2012; Wilson & Nicholson, 2017). It is clear the association between the intestinal microbiome and systemic health and nowadays, microbiome is one of the main fields of research in human and veterinary medicine (Barko et al., 2018; Cho & Blaser, 2012).

2.2 Gathering and Development of the Gut Microbiome

It was thought that in the moment of birth, the gastrointestinal tract of all mammals was sterile, being then quickly colonized by a series of microorganisms until a stable microbial community was set, around the time of weaning (Aagaard et al., 2014; Ardissone et al., 2014; Wassenaar & Panigrahi, 2014). However, some studies have shown evidence that support existence of vertical transference of microorganisms before birth (Aagaard et al., 2014; Ardissone et al., 2014; Wassenaar & Panigrahi, 2014).

The majority of the studies regarding human neonates, infer that the acquirement of the gut microbiome after birth is influenced by the organisms the neonate gets in contact from the surrounding environment and the mother (Dominguez-Bello et al., 2010; Palmer, Bik, DiGiulio,

Relman, & Brown, 2007). This theory is sustained by studies that indicate a link between the composition of the new-borns intestinal microbiome and the way of delivery in humans. Infants born by natural birth are colonized by microbial communities similar to the mother's vaginal canal, mainly composed by *Lactobacillus* spp. and *Bifidobacterium* spp. (Biasucci et al., 2010; Dominguez-Bello et al., 2010). Contrariwise, the infants born by caesarean section possess microbial communities that consist in *Staphylococcus,* microbes that are common in the skin (Dominguez-Bello et al., 2010). All these facts contribute to the theory that the acquisition of intestinal microbiome is conditioned by the organisms passed by the mother and environment in the first moments of life(Kurokawa et al., 2007; Yatsunenko et al., 2012). Also in these and other studies, it is possible to demonstrate that in what the intestinal microbiome is concerned, the initial stages of colonization are defined by a great individual and temporal variation and a more limited intra-individual taxonomic diversity (Palmer et al., 2007).

When the intestinal microbiome is set up, it will continue to evolve and will become more stable with the increase of age (Azad et al., 2013; Borody, Warren, Leis, Surace, & Ashman, 2003; Koenig et al., 2011; Palmer et al., 2007; Yatsunenko et al., 2012). This development is marked by dramatic shifts that are consequence of events and circumstances that occur in early life, as for example the shift in the food provided (Fallani et al., 2011; La Rosa et al., 2014; Palmer et al., 2007; Yatsunenko et al., 2012). Once the intestinal microbiome achieves its maturity it is expected that it will able to forage energy from complex carbohydrates, metabolize xenobiotics, and participate in the biosynthesis of vitamins (Nicholson et al., 2012; Wilson & Nicholson, 2017). However, the microbiome is not only involved in the adaption to complex nutrients, and studies in laboratory animals show its appliance in another series of processes, such as the development of the immune system, epithelium from the gut, brain and other body systems (Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014; Martinez, 2014; Olszak et al., 2012; Sommer & Bäckhed, 2015; Yang et al., 2016).

Even though the normal development of the intestinal microbiome is related to the compositional and functional changes that naturally occur during the hosts' life, there can be negative events in the early stages of life that will be associated with an abnormal maturation of the gut microbiome. Some of these negative events that can occur may be a compromised maternal health status and poor milk quality, antibiotic administration, premature birth, or even malnutrition (Arrieta *et al*., 2014; Lemas et al., 2016; Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015; Penders et al., 2006; Smith et al., 2013; Subramanian et al., 2014). Nevertheless, in what dogs and cats are regarded little is known. The only existent longitudinal study in kittens shows that, as in humans, the early microbiome is defined by a high degree of variation between individuals and an increase of stability with age of the intra-individual diversity and composition. It also reveals that the relative abundance of *Lactobacillus* and *Bifidobacterium* tend to decrease with age, and *Bacteroides* and bacterial genes that are associated with the metabolization of complex carbon sources tend to increase with age

(Deusch et al., 2015). The only study that uses DNA sequencing to evaluate the gastrointestinal microbiome in puppies, reveals a temporal instability and a great interindividual variability, meaning that the characteristics of the gut microbiome and its evolution are similar in both kittens and puppies (Burton, O'Connor, Ericsson, & Franklin, 2016).

In recent studies regarding humans and mammals, diet, along with the environment, is reported to be the primary influence on the structure and function of the gastrointestinal microbial community, having a crucial part in shaping the microbiota and affecting the short-term and long-term composition of the canine and feline gut microbiome (Bermingham et al., 2013; De Filippo et al., 2010; Deusch et al., 2015; Hang et al., 2012; Muegge et al., 2011; Wu et al., 2011). Interestingly, a large number of studies regarding the influence of the diet in the gut microbiome have been developed, mainly in both cats and dogs (Barko et al., 2018; Hang et al., 2012; J. Kim et al., 2017).

One of the studies, which aimed to compare the faecal microbial populations of kittens fed with moderate-protein diet (MP) and high-protein diet (HP), shows that at 8 weeks of age there is no difference between the kittens given the two diets, since they cluster together by litter, however at both 12 and 16 weeks of age there is a clear difference between kittens fed with MP and HP (Vester et al., 2009). It is also worth mentioning the fact that the concentrations of *Bifidobacterium*, *Lactobacillus* and *Escherichia coli* were greater in kittens given the MP at all times of the study, whereas the *Clostridium perfrigens* populations were unaffected by diet or age (Vester et al., 2009). These findings reinforce the theory that a new-born's gastrointestinal microbiome is highly influenced by the environment and family, and that later development changes in the microbiome are motivated by the diet.

There is also a study demonstrating that dietary protein/carbohydrate ratio has an effect in the development of the gut microbiome of growing kittens, especially the *Firmicutes*, *Actinobacteria* and *Fusobacteria* phyla. It also shows that a high protein diet may have a negative repercussion on the *Bifidobacterium*, *Megasphaera* and *Lactobacillus*, which are known to be health promoting bacteria. The authors also describe an association between some of the faecal bacteria families (e.g. *Lactobacillus)* and blood hormones and metabolites, which is indicative of the role they may have in the regulation of the hosts metabolism (Hooda, Vester Boler, Kerr, Dowd, & Swanson, 2013).

It is also reported that there is a difference in the gut microbiota of cats that are fed with a dry diet and the gut microbiota of cats fed with wet diet (Bermingham et al., 2013). The composition of the diet in both carbohydrates and protein also has a great influence in the abundance and variety of the gut microbiome. Consequently, there will be a bigger proliferation of the bacteria that can degrade and use the digesta quicker (Beloshapka et al., 2013; Bermingham et al., 2013; Hang et al., 2012).

Despite belonging to the order Carnivora, dogs in nature are omnivorous, and dietary carbohydrates, usually present in commercial dry and wet foods, compose their diet. Even

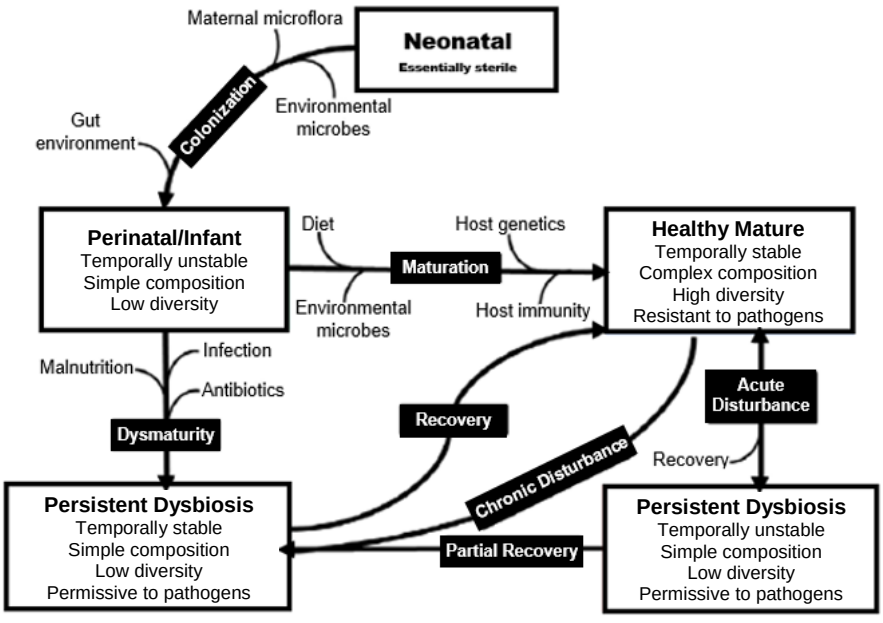
though dogs are not dependent on microbial fermentation for fulfilling energy requirements, a balanced and stable microbiota is crucial for the maintenance of gastrointestinal health (Swanson et al., 2011).

Even though these studies couldn't find the specific nutrients (macro and micro) responsible for the changes in the gastrointestinal microbiome or the mechanisms that lead to the interaction between the host and the microbe, the facts described above seem to confirm that the findings in animals are very similar to those in humans and that as the animals age, there is a development of the microbiome towards a more diverse and stable composition. It is also clear that the environment, disease states, and diet given to the animals have a preponderant role in shaping it.

There is a clear contrast between the infant and adult gut microbiome in humans since the mature microbiome is characterized by an enormous diversity in both composition and function, as well as an abiding temporal stability (Barko et al., 2018; C. A. Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). This stability over time is thought to be associated with the states of equilibrium that usually exist in the microbiota, and even though some disruption may occur the microbial community will always tend to a central point (C. A. Lozupone et al., 2012). These characteristics seem to be extended to the dog and cat gut microbiome.

Several factors have been linked to the development and resilience of the gut microbiome since the time of birth (Barko et al., 2018). Therefore, the introduction or extinction of certain microbial groups or the shift in the population structure can be caused by a diversity of events that occur during the life of the host, such as diet, environment, medical interventions (for example exposure to antimicrobials) and disease states (Barko et al., 2018; Cho & Blaser, 2012). (Figure 1)

Figure 1 - Factors that Influence the evolution and stability of the gut microbiome since birth (adapted from Barko et al., 2018)



2.3 The 16S rRNA Gene

The 16S ribosomal Ribonucleic acid gene (16S rRNA) encodes for a ribosomal subunit that is widely conserved among bacteria and contains hypervariable regions, unique to each bacterial species, interspersed among conserved regions, shared among most bacteria, of its sequence. While the hypervariable regions allow the classification or taxonomy, the conserved regions are on the base of the development of universal primers which will bind to the known sequences (Chakravorty, Helb, Burday, Connell, & Alland, 2007).

The sequencing of the 16S rRNA gene has been used in metagenomic studies in phylogenetic classifications to identify the genus and species of bacteria from a diverse number of sources, such as environmental or clinical specimens. Bacterial taxonomists have also been using it to measure DNA similarity between isolates, for a long time (Janda & Abbott, 2007; Mignard & Flandrois, 2006; Patel, 2001).

There are some characteristics that make this gene an essential phylogenetic tool, and the housekeeping gene genetic marker used in most of the situations. These characteristics include (a) it is present in almost all bacteria (b) its function not having changed over time, suggesting that changes in the sequence are more likely to reflect random changes than selected changes that could modify the function of the molecule, also, random changes are a greater measure of time (evolution) (c) the 16S rRNA gene is large enough (1,500 bp) to portray statistically important sequence information and it also consists of around 50 functional domains and nine variable regions interspersed between conserved regions. This last characteristic is very important because the higher the number of domains, the less influence the selected changes will have on phylogenetic relationships (Patel, 2001). The variable regions of 16S rRNA are used in the phylogenetic studies and the region of interest may vary depending on issues like experimental objectives, design, and sample type. (16S Metagenomic Sequencing Library Preparation, 2013)

The characteristics mentioned above are quite alluring since a single technique gives the chance to identify numerous different species and/or slow-growing, unusual and fastidious isolates or bacteria that are inadequately differentiated by conventional methods, for example, bacteria that do not fit a known biochemical profile (Janda & Abbott, 2007; Patel, 2001). The sequencing of this gene is also useful to trace phylogenetic relationships between bacteria; however studies show that it has a low phylogenetic power at the species level and a low discriminatory power for some genera (Janda & Abbott, 2007; Mignard & Flandrois, 2006). Nonetheless, this technique has some negative points, especially its need for technical resources, such as reagents and equipment for amplification and sequencing, a database with known sequences, and special software to edit sequences and database correlation (Patel, 2001).

According to some studies regarding this matter, the 16S rRNA gene sequencing is able to provide the genus identification in 90% of the cases, however regarding the species it was only

able to achieve its identification in 65 to 83% of the cases. It is also relevant the fact that 1 to 14% of the isolates remain unidentified after testing (Drancourt et al., 2000; Janda & Abbott, 2007; Mignard & Flandrois, 2006). The difficulties in identifying the genus and species of the isolates was justified by inappropriate DNA extraction, mixed culture, the recognition of novel taxa, the low number of deposited sequences in nucleotide databases and which database is used, the similarity between species and/or 16s or even nomenclature problems (Balvočiūtė & Huson, 2017; Drancourt et al., 2000; Janda & Abbott, 2007).

2.4 The Gut Microbiome Constituents

Recent metagenomic studies have described that the faecal microbiota of dogs and cats contain all three domains of life – Archaea, Bacteria, and Eukarya – being that the vast majority of the community (round 98%) consists of bacteria (Moon, Young, Maclean, Cookson, & Bermingham, 2018) (Table 1).

Table 1 - Taxonomy and phylogeny of common constituents of the gastrointestinal microbiome (adapted from Barko et al., 2018)

Phylum	Class	Order	Family	Genus
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*
			Ruminococcaceae	*Rumminococcus*
				Faecalibacterium
			Eubacteraceae	*Eubacterium*
	Bacilli	*Lactobacilliales*	*Lactobacillaceae*	*Lactobacillus*
			Streptococcaceae	*Streptococcus*
				Enterococcus
	Erysiphelotrichia	*Erisophelotrichales*	*Erysiphelotrichaceae*	*Turicibacter*
				Catenibaterium
				Coprobacillus
				Allobaculum
	Negativicutes	*Selenomonadales*	*Selemonadaceae*	*Megamonas*
		Veillonellales	*Veillonellaceae*	*Dialister*
				Megasphaera
				Villonella
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*
			Bacteroidaceae	*Bacteroides*
Actinobacteria	*Coriobacteriia*	*Coriobacteriales*	*Coriobacteriaceae*	*Colinsella*
			Atopobiaceae	*Olsenella*
		Eggerthellales	*Eggerthellaceae*	*Slackia*
				Eggerthella
	Actinobacteria	*Bifidobacteriales*	*Bifidobacteriaceae*	*Bifidobacterium*
Fusobacteria	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteraceae*	*Escherichia*
				Shigella
		Aeromonadales	*Succinivibrionaceae*	*Succinivibrio*
				Anaerobiospirillum

In the intestinal tract of healthy dogs and cats the bacterial phyla that prevail are *Firmicutes* and *Bacteroidetes*, as in humans, followed by *Fusobacteria*, *Proteobacteria* and *Actinobacteria* (Moon et al., 2018). The most abundant genus observed in canine and feline faeces is the *Clostridium*, fluctuating between 7-22% (Garcia-Mazcorro, Dowd, Poulsen, Steiner, & Suchodolski, 2012; Hand, Wallis, Colyer, & Penn, 2013; Handl, Dowd, Garcia-Mazcorro, Steiner, & Suchodolski, 2011; Kerr, Forster, Dowd, Ryan, & Swanson, 2013; Swanson et al., 2011). Not similarly to humans or even other mammalian species, the *Fusobacteria* phylum is highly represented in canine faeces and in some studies it was even the dominant phylum or had the highest percentages of abundance alongside the *Firmicutes* and *Bacteroidetes* (Hand et al., 2013; Kerr et al., 2013; Middelbos et al., 2010). Nevertheless, there are some differences among the existing publications regarding the relative abundance of each phyla, this may be explained by the differences in the individual host genetics, diet, age, method used for sample collection as well as the source of the sample, DNA extraction techniques or even the analytic method used in each study (Barko et al., 2018; Caporaso et al., 2012; Kennedy et al., 2014; Luo, Tsementzi, Kyrpides, Read, & Konstantinidis, 2012).

It has been assumed that the general roles and functions of these bacterial phyla in the dog and cat are similar to those in the humans, however some evidence that contradicts this assumption has been rising (Moon et al., 2018). An example is the *Fusobacterium* which has been linked with inflammatory bowel disease and colorectal cancer in humans, but not in dogs, where they have been identified in high abundances in healthy animals fed with raw meat (Ley et al., 2008; Vázquez-Baeza, Hyde, Suchodolski, & Knight, 2016).

The faecal microbiota of healthy dogs and cats commonly carries *Proteobacteria*, the most diverse bacterial phylum, being that its most prevalent class in the gastrointestinal tract are the *Gammaproteobacteria*. It is hypothesized that the *Proteobacteria* play a key role in the maintenance of the homeostasis of the anaerobic environment of the gut. However, in this phylum are included some of the most well-known opportunistic pathogens, such as *E. coli, Klebsiella pneumoniae, Salmonella,* and *Campylobacter*, which are known to have a negative impact in the health of the host (Moon et al., 2018).

Based on a study using 16S rRNA gene pyrosequencing for evaluation of faecal microbiota in healthy dogs and cats, *Firmicutes* was the most copious bacterial phylum in both species. *Clostridia* the most predominant bacterial class, and *Clostridium* and *Ruminococcus* its dominating genera. Along with *Clostridia*, the *Bacilli* and *Erysipelotrichi* classes were quite representative, being the first one composed almost solely by the *Lactobacillales* order in both dogs and cats. This order was mainly represented by the genera *Streptococcus* and *Lactobacillus* in dogs, and *Enterococcus* and *Lactobacillus* in cats. The *Erysipelotrichi* class was exclusively composed by the *Erysipelotrichales* order. By its turn, this order consisted of the genera *Turicibacter*, *Catenibacterium*, and *Coprobacillus.* In dogs, the second most representative phylum was *Bacteroidetes*, containing the *Prevotella*, *Bacteroides*, and

Megamonas genera. In cats, the second most abundant phylum was *Actinobacteria*. This study also points that the cat faecal microbiota seems to be more diverse than the dog faecal microbiota (Handl et al., 2011). Following this idea, the majority of the studies describe the gastrointestinal microbiome from cats as quite similar in composition to dogs and other mammals. In cats, *Firmicutes* is the most abundant phylum, having a relative abundance of about 36-92% (Handl et al., 2011; Ritchie, Burke, Garcia-Mazcorro, Steiner, & Suchodolski, 2010; Ritchie, Steiner, & Suchodolski, 2008). *Clostridiales* is the most abundant order and *Clostridium* its most representative genus (Handl et al., 2011; Ritchie et al., 2010, 2008). In contrast with the canine gut microbiome composition, in felines, the *Proteobacteria* constitute a considerable percentage of the gut microbiome, having a relative abundance ranging from 7.9 to 14% (Barry et al., 2012; Ritchie et al., 2010, 2008).

Even though nearly all the studies in dogs and cats focus on the characterization of the faecal microbiome as a whole, it is important to mention that there is quite a difference between the microbiome's composition of the various intestinal segment (Ritchie et al., 2008; Suchodolski, Camacho, & Steiner, 2008). Up to date, there are two articles published regarding this matter, one concerning the feline intestinal tract and other concerning the canine's one. In cats, as seen in other studies, most of the clones (82%) were classified as belonging to the *Firmicutes* phylum, *Clostridia* class, followed by *Bacilli* class being that from this last, 48% were isolated from the jejunum and 39% from the colon and 13% in the ileum and less than 2% in the jejunum. The second most represented phylum was the *Proteobacteria*, 41% of the clones were isolated from the duodenum followed by the ileum (32%), colon (19%), jejunum (5%) and rectum (3%). The third most isolated phylum was *Bacteroidetes,* most of the clones were isolated from the colon (50%) and ileum (43%), followed by the rectum and jejunum (5% and less than 2%, respectively) (Ritchie et al., 2008). Comparing each segment, the small intestine microbiome was dominated by *the Firmicutes* and *Bacteroides*, whereas the ileum was mostly composed by *Proteobacteria* and *Actinobacteria*; the *Firmicutes*, *Proteobacteria* and *Fusobacteria* were the predominant phylum in the colon (Ritchie et al., 2010). Regarding the dogs the study shows that there is a gradual increase in the bacterial diversity along the intestinal tract, from the duodenum to the colon. In the duodenum and jejunum microbiome the most abundant order was the *Clostridiales*, whereas in the ileum and colon the most abundant bacterial orders were *Fusobacteriales* and *Bacteroidales*, respectively. Organisms belonging to the *Proteobacteria* phylum (including *E. coli* organisms) take a huge part in the duodenal microbial community (32%), however it is not as substantial in the colon, representing only 1.4% of its constituents. The order *Lactobacillales* was well represented in the duodenum, jejunum and colon, however they were only present in a small fraction in the ileum (1.4%) (Suchodolski et al., 2008).

As mentioned before, the genetics of the individual seems to influence the composition of the gastrointestinal microbiome; however, it is still uncertain how it happens. Some studies in

humans based on 16S rDNA sequencing indicate that related humans have a more similar composition of the microbiome than unrelated humans and that heritability of the gut microbiome is correlated with the specific host gene elements (Goodrich et al., 2016; Khachatryan et al., 2008; Turnbaugh et al., 2009). It is also likely that the microbiome is shaped accordingly to the hosts diet preference, which is also inherited (Goodrich et al., 2016). Likewise, faecal microbiomes of genetically related dogs seem to be more similar between each other than those of unrelated dogs (Hand et al., 2013). Another study comprehending a large number of humans, including members of the same family, and even twins, reveals that there was no difference in the similarity levels of the gut microbiome between individuals belonging to the same family, and unrelated individuals residing in the same geographic and cultural region (Yatsunenko et al., 2012). The same study also states that people based in different regions can be put apart from one another by comparing their intestinal microbiome's composition and genomic features (Yatsunenko et al., 2012).

Regarding the metabolic capacity of the canine gut, the bacterial functional gene categories that are most representative, include carbohydrate metabolism (12–13% of all sequences), protein and amino acid metabolism (8–9 and 7%, respectively), cell wall synthesis (7–8%), vitamin and cofactor synthesis (6%), and nucleic acid synthesis (7%) (Swanson et al., 2011). According to a study focused on the metagenomic analysis of the feline intestinal microbiome, the values of microbial carbohydrate and protein metabolism (13.33% and 8.99% respectively) and DNA metabolism were similar to the described previously for canine metagenome. However, the feline metagenome seems to present a higher percentage (5.77%) of genes associated with RNA metabolism (Tun et al., 2012). The metagenomic analysis of the feline and canine microbial community also demonstrate the existence of genes associated with the resistance to antibiotics and toxic compounds (Swanson et al., 2011; Tun et al., 2012).

Therefore, the feline and canine microbiota have a direct influence in their hosts health and in human health as they may be reservoirs for antibiotic resistant bacterial strains (Swanson et al., 2011; Tun et al., 2012).

Besides the diet and environment, the medical interventions the individual suffers during his life will have an important role in shaping the gut microbial community. For instance, the administration of ciprofloxacin will reduce the taxonomic diversity and richness of the human faecal microbiome (Dethlefsen, Huse, Sogin, & Relman, 2008; Dethlefsen & Relman, 2011). After the course of medication, the recovery of the microbiome is only partial and its characteristics don't return to the pre-antimicrobial state, resulting in a new, long-term compositional steady state (Dethlefsen & Relman, 2011; Suchodolski et al., 2009). A similar study, focusing on the macrolide administration in dogs, demonstrates that this drug induces a compositional diversity and richness decrease in the faecal microbiome of this specie. The response to treatments with tylosin is different between individual animals, however, a persistent reduction in jejunal microbial diversity and richness was observed in 40% of the

dogs (Suchodolski et al., 2009). It is also proved that antimicrobials are responsible for the changes in the composition of the gastrointestinal microbiota (Morgan et al., 2012). And some assays indicate that the administration of antimicrobials, such as metronidazole, can slow down the recovery from dysbiosis, and that sick dogs that did not go through an antimicrobial treatment course have bacterial groups in more similar quantities to healthy dogs (Minamoto et al., 2015). Besides antimicrobials, other medications, such as pump proton inhibitors (omeprazole), can affect the microbial populations in the canine gastrointestinal tract (Garcia-Mazcorro et al., 2012). In this study, it was demonstrated an increase in duodenal bacterial and changes in the faecal microbiome after the treatment with omeprazole. The shifts in the faecal microbiome were characterized by an increment of the relative abundance of *Lactobacillus* and a decrease in the proportions of *Faecalibacterium* and *Bacteroides*. This shift is probably associated with the increase of the bacterial load entering the large intestine or the modification in the composition of the dietary protein that reaches the large intestine (Garcia-Mazcorro et al., 2012).

The studies mentioned along this section seem to indicate that, as in humans, adult dogs and cats have an intestinal microbiome characterized by its long-term stability and compositional and functional diversity. Another important assessment is the clear interaction between the host and it gut microbiome which will affect the vital physiological processes in the hosts body, and that any condition or event that may disturb the microbiome can have a negative impact in the host health. These features of the microbiome make it a viable and helpful target for diagnostics and therapeutics of certain diseases (Barko et al., 2018).

2.5 Gut Microbiome in Health and Disease

Once set, the microbiome is extraordinarily varied and resilient, and the microorganisms cooperate in dynamic and symbiotic equilibrium with the host cells, making key contributions for the protection of the organism against enteropathogens, maintenance of energy homeostasis and metabolism, synthesis of vitamins and other nutrients, endocrine signalling, gut epithelial health, immunologic activity and neurobehavioral development (Cho & Blaser, 2012; Shreiner et al., 2015; Turnbaugh et al., 2007). Lately, there has been an association between several gastrointestinal and systemic diseases with abnormal gut microbial communities. However, despite the array of evidence that supports the interaction between the microbiome and the host metabolic and immune systems, it is still unclear if there is a real association between them (Barko et al., 2018).

The host and its gastrointestinal microbiome coexist as mutualists, however in some situations this relation becomes pathological as in obesity, diabetes, atherosclerosis and inflammatory bowel diseases (IBD) (Wu et al., 2011).

Hosts health is largely influenced by the diversity of the intestinal microbiome, and a decrease in diversity walks hand in hand with a panoply of gastrointestinal and systemic diseases in both

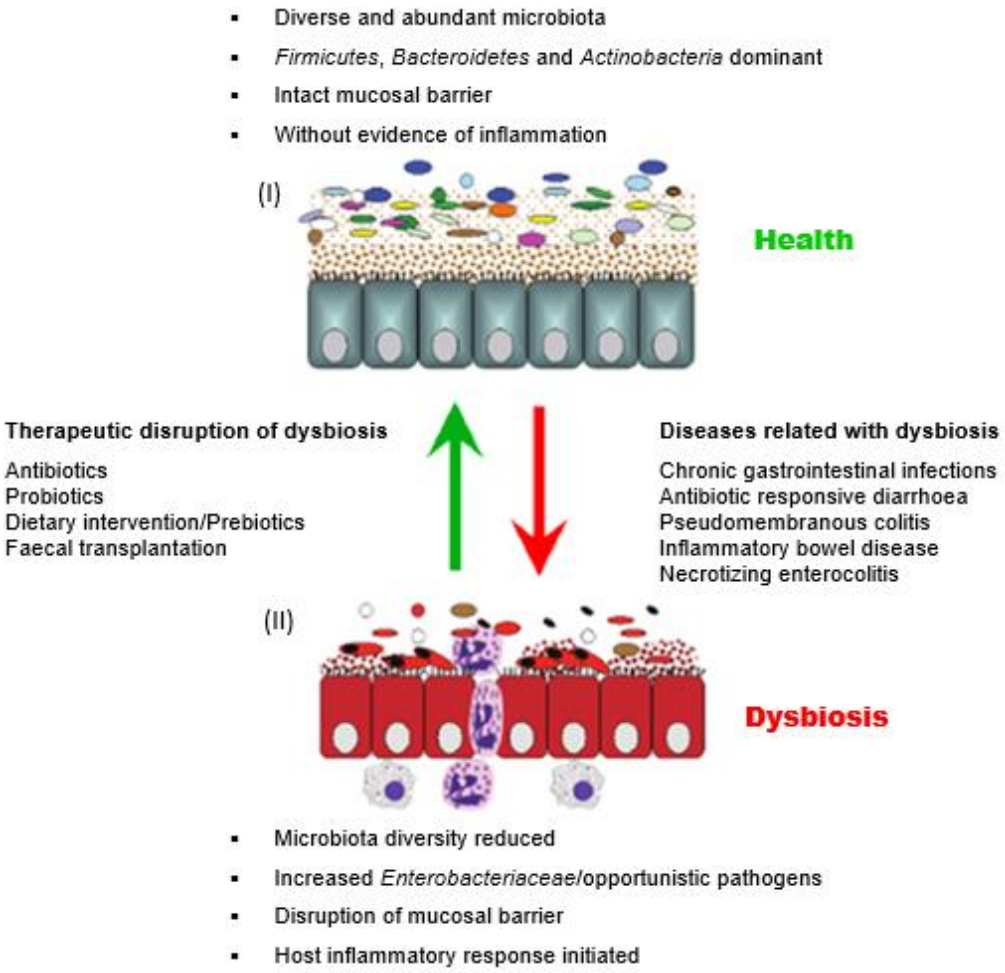
humans and other mammals (Frank et al., 2007; Ianiro, Tilg, & Gasbarrini, 2016; Ley et al., 2005; Tong et al., 2013; Turnbaugh et al., 2006). The balance that exists in bacterial populations of the gut microbiota is extremely delicate, therefore, a disturbance of its balance will lead to antibiotic responsive diarrhoea, previously referred as dysbiosis, and a consequent decrease in resistance to colonization by pathogens (Kamada, Seo, Chen, & Núñez, 2013).

Intestinal dysbiosis (Figure 2) is characterized by a change in the intestinal microbiome with a rearrangement of microbial compositional structures, decrease in diversity and shifts of the relative proportion of certain organisms. Dysbiosis will favour the development of pathogenic features which will affect the health of the host organism (Frank et al., 2007; Kamada et al., 2013).

The origin for the antibiotic responsive diarrhoea is different among individuals and is influenced by the pathological condition that is affecting them. However, there are some common characteristics of dysbiosis in both humans and laboratory animals, such as the decrease in the relative proportion of obligate anaerobic bacteria of the *Firmicutes* phylum and the increase presence of facultative members of the Enterobacteriaceae family, including *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Proteus* spp. and *E. coli* (Walker & Lawley, 2013).

A study in dogs with chronic enteropathy has shown a significantly decrease in bacterial diversity and different microbial communities in these animals when compared with healthy dogs (Minamoto et al., 2015). In dogs that presented the disease, the *Erysipelotrichia*, *Clostridia*, and *Bacteroidia* classes were underrepresented, while the *Gammaproteobacteria* were overrepresented, mainly due to the increase in *Enterobacteriaceae* (Minamoto et al., 2015). A factor that seems to contribute to the persistent dysbiosis in some dogs with IBD is the administration of certain antimicrobials, such as metronidazole (Minamoto et al., 2015). After treatment, it was registered a clinical improvement in all dogs with IBD, however, there was not a significant change in the faecal microbiota. This fact suggests the existence of a functional change of the gut microbiota in dogs with IBD that persists even after medical treatment and clinical recovery. Consequently, dogs with persistent dysbiosis will probably need additional therapy and/or a more prolonged treatment course. Furthermore, these dogs seem to have a higher risk of relapsing clinical signs (Minamoto et al., 2015).

Figure 2 – Human intestinal health and dysbiosis. (I) Healthy intestine is defined by a varied and rich microbiota, mainly composed by members of the Firmicutes, Bacteroidetes and Actinobacteria phyla. (II) Dysbiosis is characterize by a reduction of bacteria diversity (adapted from Walker & Lawley, 2013).



Another outsanding problem in veterinary medicine is the escalating numbers of obese animals. This fact is important since there are some recent studies that reveal an association between obesity and intestinal microbiome in humans, laboratory animals and pets(Ley et al., 2005). The mechanisms that seem to be involved are the increase in dietary energy harvest, changes in glucose and lipid metabolism induced by microbes, insulin resistance caused by chronic low-grade inflammation and microbial signalling through host endocrine systems (Ley, 2010; Li, Lauber, Czarnecki-Maulden, Pan, & Hannah, 2017).

A study in obese and lean cats indicates that there is a link between obesity and the changes in gut microbiota, however it was not possible to find an association to a specific bacterial taxa (Kieler, Mølbak, Hansen, Hermann-Bank, & Bjornvad, 2016). On the other hand, a study comparing the faecal microbiota of lean and obese dogs found no major difference in the composition of faecal microbial composition between these two groups (Handl et al., 2013).

In the same way to what is now observed in Human Medicine, in which the gut microbiome has been used as a diagnosis and therapeutic tool for several diseases such as asthma, autism, diabetes, obesity and many others, we do believe that in Veterinary Medicine the gut microbiome may be the key for the diagnosis and therapy of several important diseases.

3 Study – Enteric microbiome in healthy companion animals

3.1 Objectives

The gut microbiome in companion animals has been one of the recent focuses of veterinary research as it may be the key to understand several disease states as well as for new therapeutic approaches to these diseases. However, little is known about the microbiome and the bacteria that compose it in healthy companion animals. This study aims to clarify the gut microbiome of healthy dogs and cats contributing for a better understanding of the effect that environment can have on it.

In this study, we focused in studying the gut microbiome of healthy companion animals in order to understand how is the normal microbiome without the influence of metabolic disarrangements, disease situations or antimicrobials intake. We also decided to compare two different groups of animals, taking in account the conditions they were presently living. The first group includes the household companion animals with more human contact (living in human households) and the second group, which includes the shelter cats and dogs that have much less contact, very less medical interventions and a different environment influence. Therefore, the goal of this study is to understand the differences in microbiome diversity between:

- **Dogs** from shelter and dogs from households
- **Cats** from shelter and cats from households

3.2 Material and Methods

3.2.1 Animal selection

For this study two groups of animals were selected, a group composed by both dogs and cats that belong to a household and have closer contact with humans (household groups) and a group of animals, also dogs and cats, that belong to two official shelters (shelter groups). The samples were collected between 2016 and 2017. Both the owners of the animals and the veterinarian responsible for the shelter gave their consent for the sample collection and use for this study. Companion animals were sampled without using any invasive methods. In the household group, a total of 38 animals were included in the study, 26 dogs and 12 cats. The shelter group consisted of 62 animals, 51 dogs and 11 cats.

Background information of the animals from both groups was collected, including: age, gender, breed, previous medications with antimicrobials, probiotics and prebiotics, proton pump inhibitors or prednisolone. For the shelter group it was also asked the date of their arrival. Animals that were receiving or had received antimicrobials or had been hospitalized in the previous month were excluded from the study and if they suffered an episode of vomiting or diarrheal disease in the last 1 month prior to the study. Also, the females included in this study were not pregnant or lactating at the time of faecal sample collection. All the animals were

eating a variety of commercial dry food and hadn't taken any probiotic or prebiotic. All the shelter animal at to be living there for at least one month.

The household dogs (16 males and 10 females) had ages ranging between 2 months and 17 years old. Three animals were puppies (< 18 months), five animals were young adult (18 months – 3 years old), fifteen animals were adult (3 years old – 10 years old) and three animals were at the senior stage (> 10 years old). Concerning the animal breeds they were Yorkshire Terrier (*n*= 7/26), mixed breed (*n*= 6/26), Labrador Retriever (*n*= 3/26), Dachshund (*n*= 2/26), Toy Poodle (*n*= 2/26), French Bulldog (*n*= 1/26), Miniature Pinscher (*n*= 1/26), West Highland White Terrier (*n*= 1/26), Brittany dog (*n*= 1/26), American Akita (*n*= 1/26) and Pekingese (*n*= 1/26). As for the shelter dogs (30 males and 21 females), the ages ranged between 2 months and 14 years old. Twelve animals were puppies, twenty-one animals were young adult, fifteen animals were adults and three animals were at the senior stage. Most of the animals were mixed breed (*n*= 41/51), the other breeds were Portuguese Podengo (*n*= 3/51), Labrador (*n*= 2/51), St. Bernard (*n*= 2/26), Miniature Pinscher (*n*= 1/51), German Shepherd (*n*= 1/26) and Serra da Estrela (*n*= 1/26).

Regarding the 11 cats belonging to the household group (8 males and 3 females), the ages ranged between 2 months and 17 years old. Two animals were kitten (< 6 months), five animals were young adults (7 months - 2 years old), two animals were adult (3 - 6 years old), one was mature (7 - 10 years old) and one cat was considered geriatric (> 15 years old). As for the breeds of the animals, 10 cats were Domestic short-hair and one was Persian. The shelter cats (6 males and 5 females) had ages between 2 months - 2 years old. Five of the animals were kitten and six were young adult. All the shelter cats were Domestic short hair.

3.2.2 Faecal Samples Collection

The sample collection method was equal for each group and consisted of a faecal sample collected directly into a sterile container, sent immediately to the Laboratory of Antimicrobial and Biocides Resistance of the Faculty of Veterinary Medicine - University of Lisbon where they were refrigerated (4 ºC) until they were processed within 24 hours.

Once in the laboratory, each faecal sample was mixed in sterile conditions, in order to include both internal and surface content and avoid contamination. For each sample, an aliquot was stored in a sterile tube and preserved at -80 ºC until further genomic DNA extraction.

3.2.3 Faecal genomic DNA extraction

Genomic DNA (gDNA) extraction was done directly from the faeces using the PowerSoil® DNA Isolation Kit from MoBio (MO BIO Laboratory Inc., Carlsbad, CA) according to manufacturers' instructions. The choice of the extraction kit was made based on the Human Microbiome Project (Turnbaugh et al., 2007). Extracted DNA was eluted in 100 µl of sterile elution buffer (10 mM Tris). Empty PowerBead tubes were use as negative controls of DNA extraction. After

the elution, the extracted DNA was verified for purity (based on UV absorption spectrum and 260/280 nm and 260/230 nm absorption ratios) and quantified on a ND200c spectrophotometer (Nanodrop Technologies Inc).

3.2.4 16S rDNA sequencing

The 16S rRNA gene amplification and sequencing were performed at the Instituto Gulbenkian da Ciência (IGC) - Genomic Unit.

The used metagenome protocol for metagenomic sequencing targets the V4 region of the 16S rRNA gene (primers 515f-806r) of the 16S rRNA gene and pair-end, amplifying both bacteria and archea domains (Caporaso et al., 2012; Walters et al., 2016). The sequencing was executed on an Illumina MiSeq sequencer.

3.2.5 Data analysis

The raw sequences were treated using QIIME2 (Quantitative Insights into Microbial Ecology) analysis package, version q2cli 2018.6.0 (available at https://qiime2.org) analysis package to perform quality filtering, definition of operational taxonomic units (OTUs) and taxonomic classification. The Greengenes database (v13_8) (DeSantis et al., 2006; McDonald et al., 2012) was chosen for taxonomic classification alignment at 99% similarity. The quality scores plots for the raw sequencing data were filtered for a minimum quality of 15 with DADA2 tool (Callahan et al., 2016).

The Kruskal-Wallis nonparametric test for multiple pairwise comparisons was applied to compare the Alpha diversity (relative abundances of bacterial taxa) of the three groups (with human contact, without human contact, and negative control group).

Beta diversity (bacterial community composition) within the same 3 groups was calculated using the permutational multivariate analysis of variance (PERMANOVA) test to measure the similarity between samples. Principal coordinates (PCoA) and bar plots of samples using the unweighted unifrac distance metric (C. Lozupone, Hamady, & Knight, 2006) were obtained using QIIME 2 view tool.

The Analysis of Composition of Microbiomes (ANCOM) method was used to evaluate if the differences of taxa abundance observed in the taxonomic composition (phylum, classes and genera) of animals with and without contact were statistical significant, W value was determined (Mandal et al., 2015). The relative abundances of reads for phylum, classes and genera were determined by using QIIME taxa collapse, QIIME composition add-pseudocount and QIIME composition tools. A p-value < 0.05 was considered statistically significant.

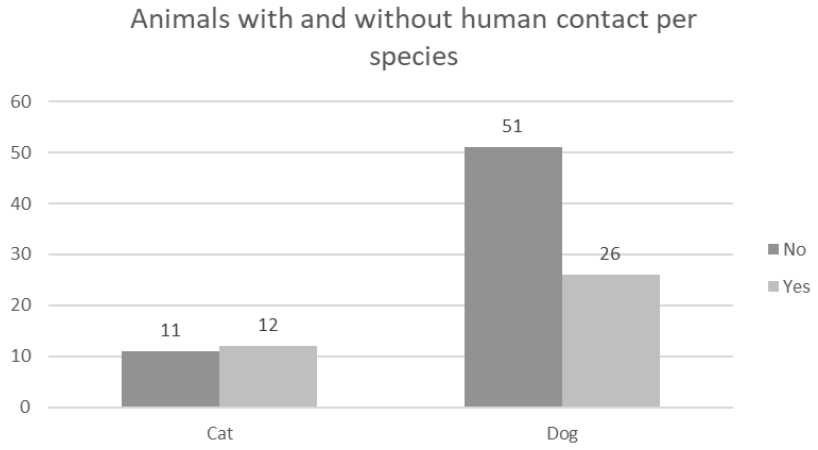
SAS statistical software package for Windows (v9.4, SAS Institute Inc, Cary, North Carolina, USA) was used. Fisher's exact test was performed to compare the faecal bacterial community proportions between dogs with and without contact. A p-value < 0.05 was considered and the Bonferroni p value adjustment method was used to account for multiple comparisons.

3.3 Results

The original sample collection contained 101 samples, 23 from cats (11 from shelter and 12 were from households) and 77 from dogs (26 from households and 51 from shelter) (figure 3). Three samples, two dogs and one cat, all belonging to the household group, had to be removed due to the lack of sequencing data, bringing the total to 11 cat samples without human interaction and 11 cat samples with human interaction, and 51 dog samples without human interaction and 24 samples with human interaction.

The household group had animals with ages ranging from 2 months to 17 years old and the shelter group with ages ranging from 2 months to 14 years old. Regarding the gender of the animals, the first group was composed by 24 males and 14 females, the second group had 27 males and 35 females.

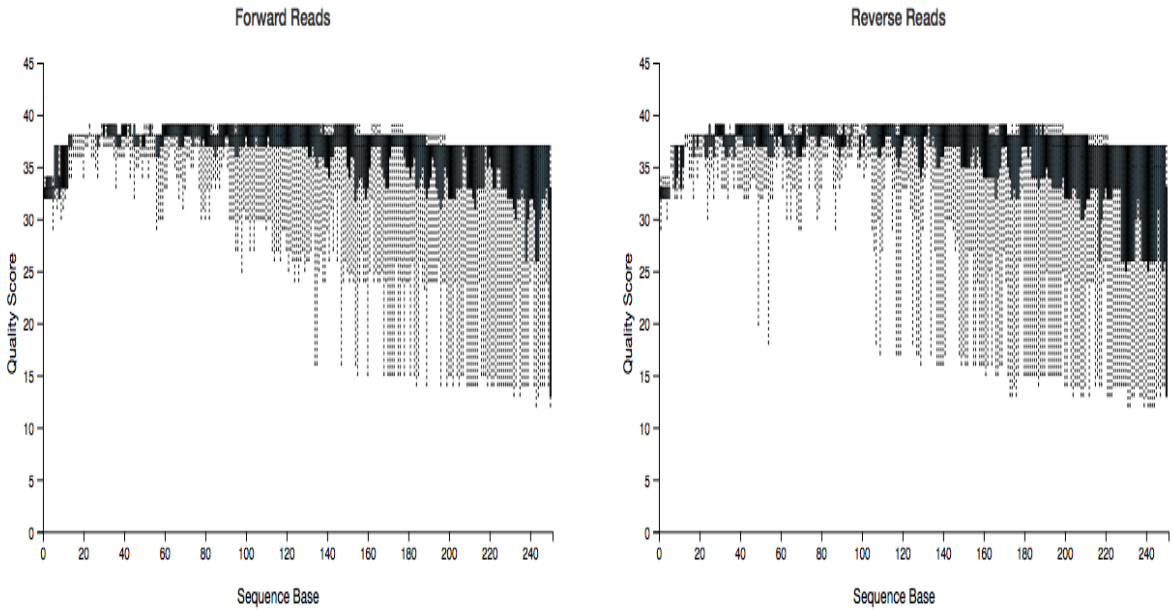
Figure 3 - Number of samples per specie (dog and cat), with and without human interaction.



3.3.1 Raw Data

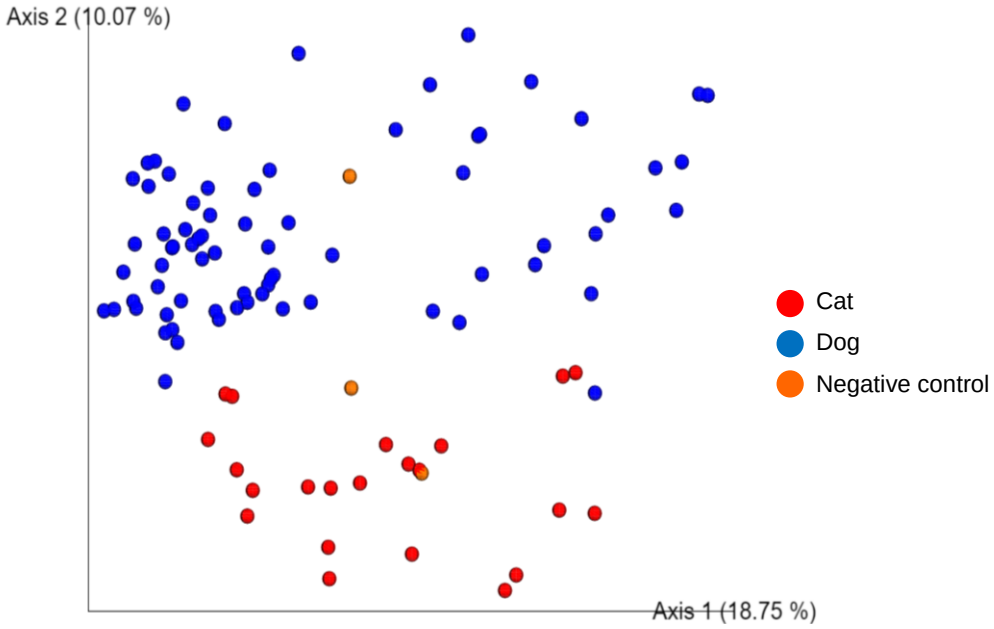
Quality control scores plots (Figure 4) were generated using a random sampling of 10000 out of 3873592 sequences without replacement. The minimum sequence length identified during subsampling was 250 bases. The data was filtered for a minimum quality of 15 and the samples were rarefied to 1 302 read depth in according to the sample with the lowest read depth.

Figure 4 - Quality scores plots for the forward and reverse read data



The PCoA plot obtained shows that the dogs and cats are fairly well separated however (Figure 5).

Figure 5 - Principal coordinate analysis (PCoA) plot coloured by species (cat, dog and negative controls) with the respective Scree Plot



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Four negative controls were made along the study. In three of the negative controls there was identification of some bacteria phyla, mainly *Firmicutes*, *Bacteroidetes* and *Fusobacteria*, and also a small percentage belonging to an unassigned domain.

3.3.2 Analysis of the Dog Samples

The quality-controlled feature table obtained with DADA2 was filtered to include only the 75 samples belonging to “Dog” and the 4 negative controls. (Figure 6, Figure 7) Besides the “negative_control_4”, “S11a1” and “C31” samples that had 0 sequence counts, the minimum sequence count is 1 302, belonging to the “negative_control_5” sample. The sample with highest sequence count is C12, with 42 046 sequences.

Figure 6 - Sample distribution per species.

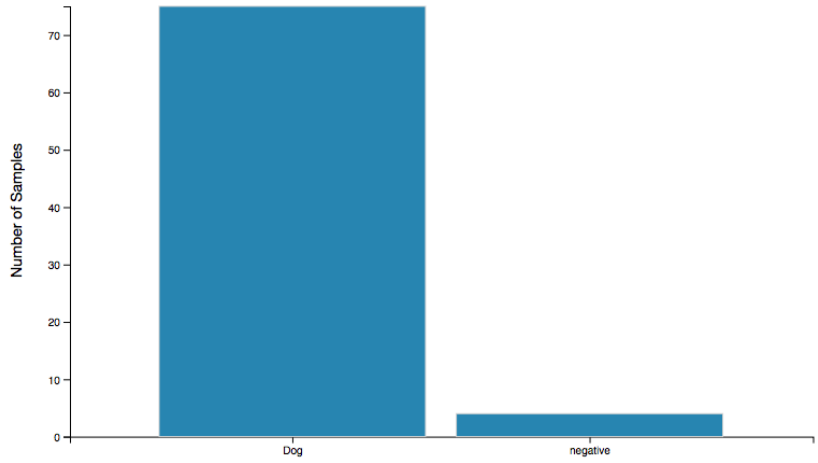
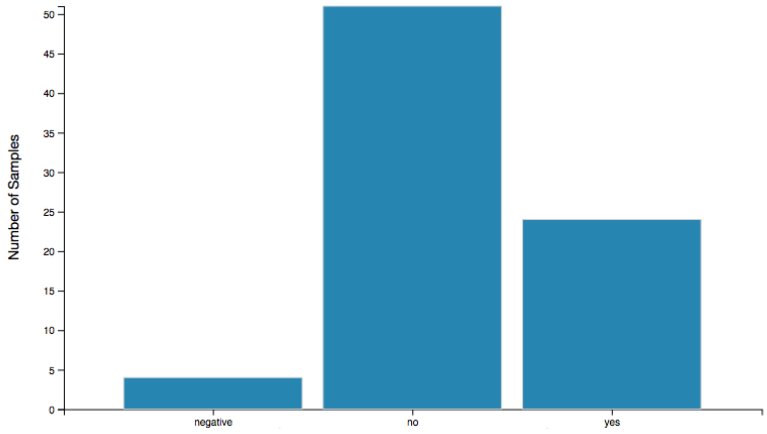


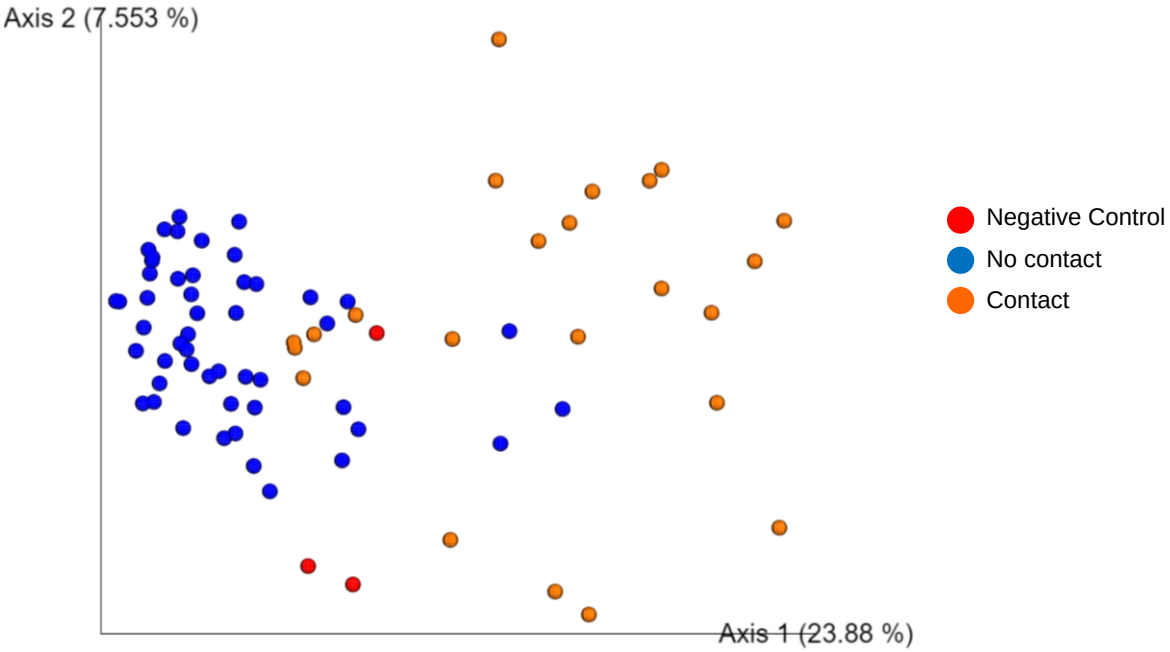
Figure 7 - Sample distribution per human contact.



The Alpha and Beta diversity between the groups that have and do not have human contact was calculated and the samples were rarefied to 1 302 read depth in according to the sample with the lowest read depth.

The PCoA plot was obtained and as it possible to observe, the dogs with and without human contact seem fairly well separated, which is an important fact to assess (Figure 8).

Figure 8 - PCoA plot (unweighted unifrac) for the dog and negative control samples coloured by human contact (yes, no and negative controls) with the respective Scree Plot.



Alpha Diversity:

The alpha diversity, i.e. taxonomic diversity within a sample, for all groups obtained a p-value <0.00001 in the Kruskal-Wallis nonparametric test, which means that there is statistical significant difference in the organismal richness of the sample and the evenness of organisms' abundance distribution (Table 2). It is also possible to observe, in the boxplots, that there is a clear difference between the dogs in general and the negative controls (Figure 9) and that the shelter group had a higher number of observed operational taxonomic units (OTUs) than the household group (p-value <0.00001) (Figure 10, table 2).

Figure 9 - Alpha diversity boxplots for the dog and negative control samples

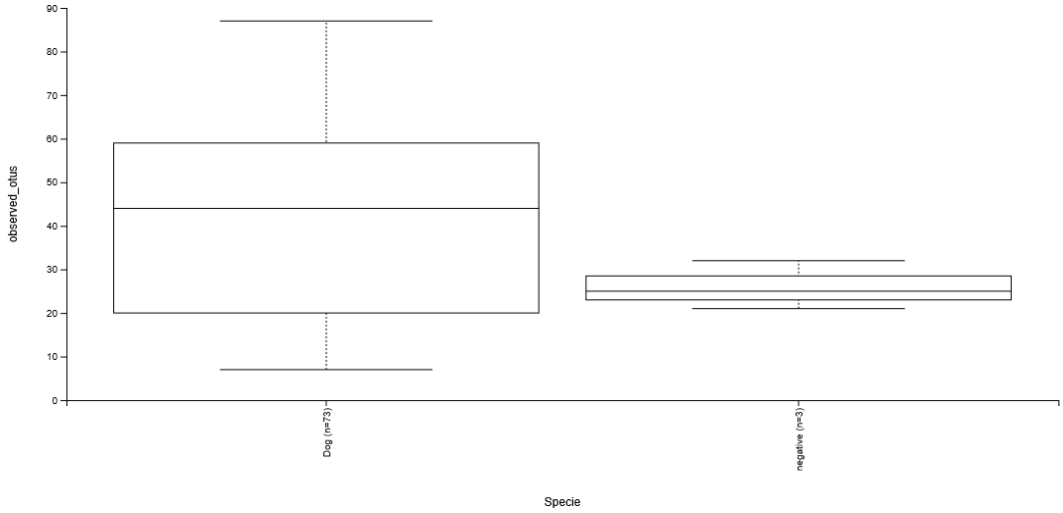


Figure 10 - Alpha diversity boxplots for the dog and negative control samples according to human contact (yes, no and negative control)

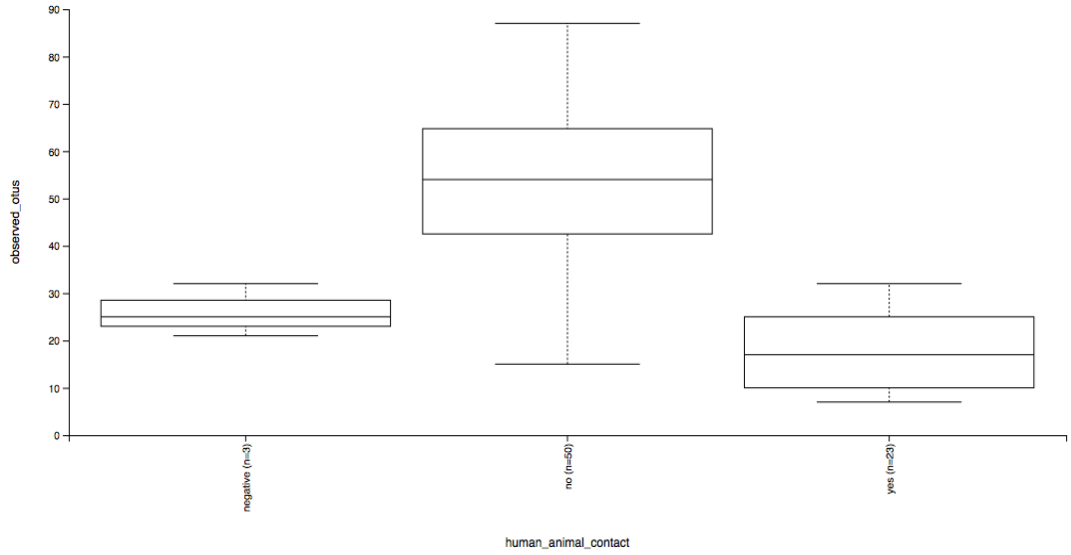


Table 2 Pairwise Kruskal-Wallis test for the dog and negative control samples according to human contact (yes, no and negative control)

		H	**p-value**	**q-value**
Group 1	**Group 2**			
negative (n=3)	no (n=50)	5.608308	1.787552e-02	2.661328e-02
	yes (n=23)	2.860136	9.079981e-02	9.079981e-02
no (n=50)	yes (n=23)	39.264331	3.701417e-10	1.110425e-09

Beta Diversity:

Beta diversity, i.e. taxonomic diversity between samples, analysis was used to determine the faecal microbiome composition diversity between the animals using the Principal coordinate analysis (PCoA) plot based on the unweighted – unifrac distance. To test differences in mean community structure the PERMANOVA method and pseudo-F tests were performed and statistical significance was observed between the 3 groups (p-value =0.001) (Figure 11, Figure 12, Figure 13, Table 3).

Table 3 - PERMANOVA test results for the dog samples

Sample size	76
Number of groups	3
Test statistic	8.99757
p-value	**0.001**
number of permutations	999

Figure 11 - Box plot of the distance the negative control, dogs without human contact and dogs with human contact to the negative control.

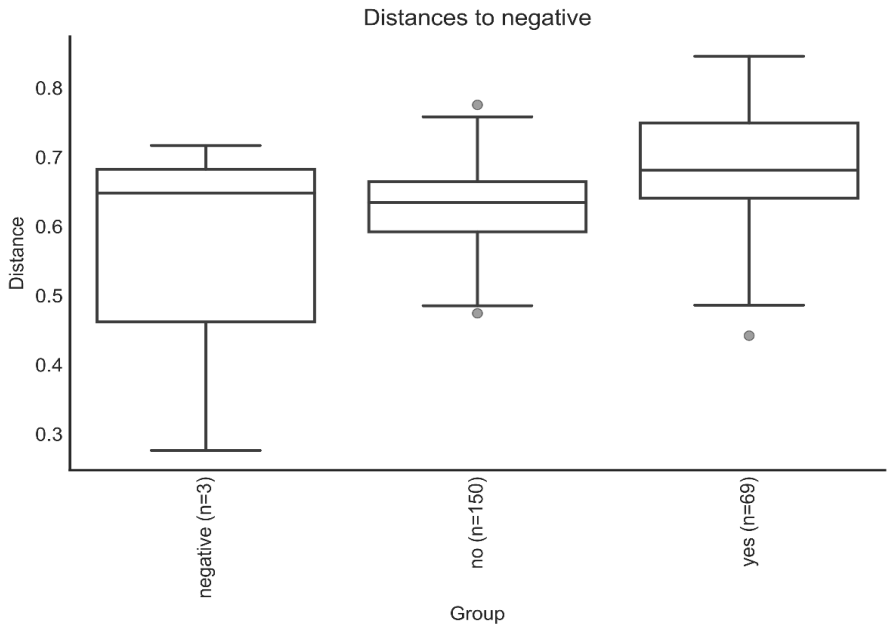


Figure 12 - Box plot of the distance the negative control, dogs without human contact and dogs with human contact to the dogs without human contact.

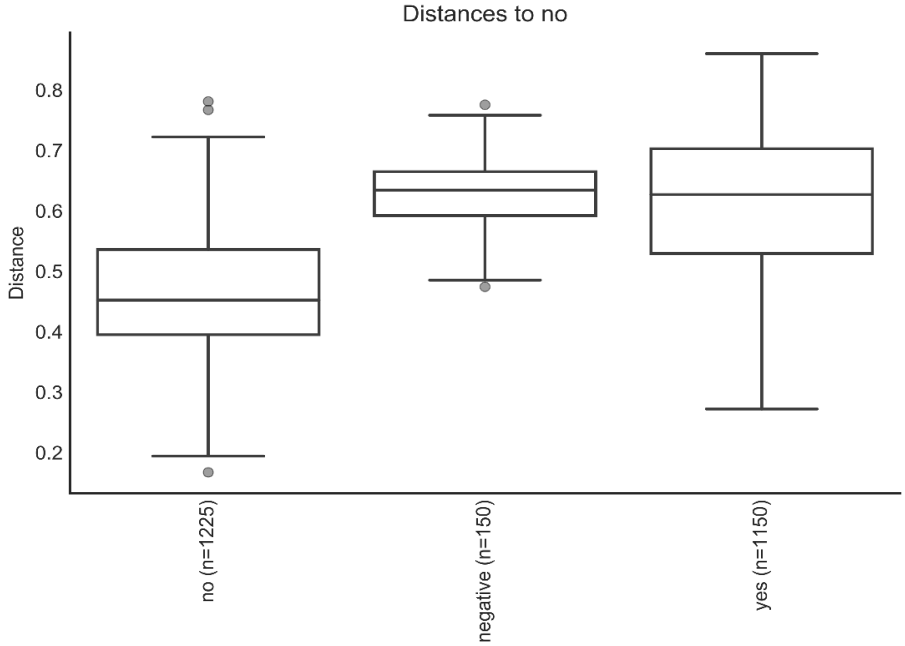
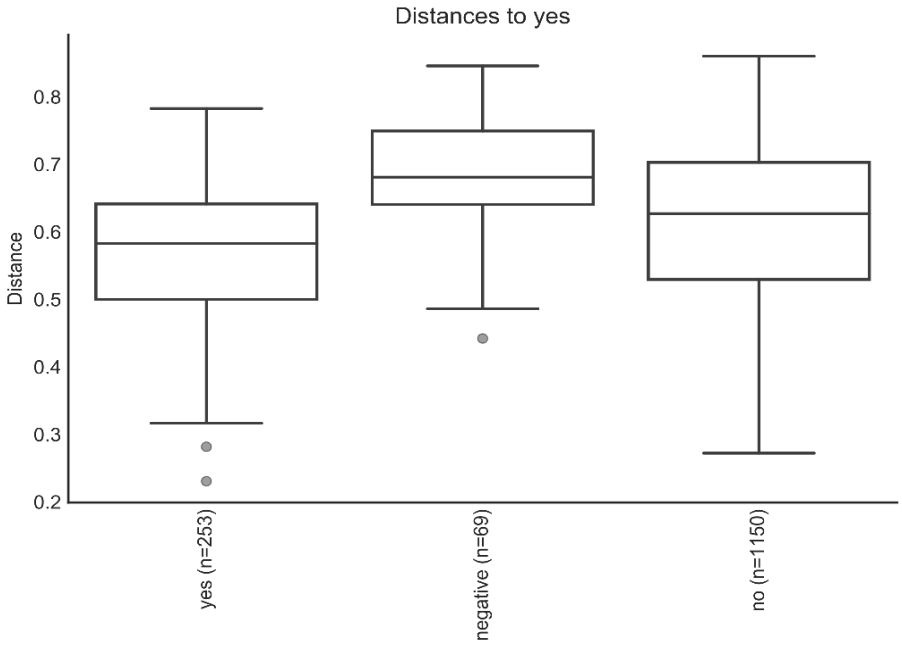


Figure 13 - Box plot of the distance the negative control, dogs without human contact and dogs with human contact to dogs with human contact.

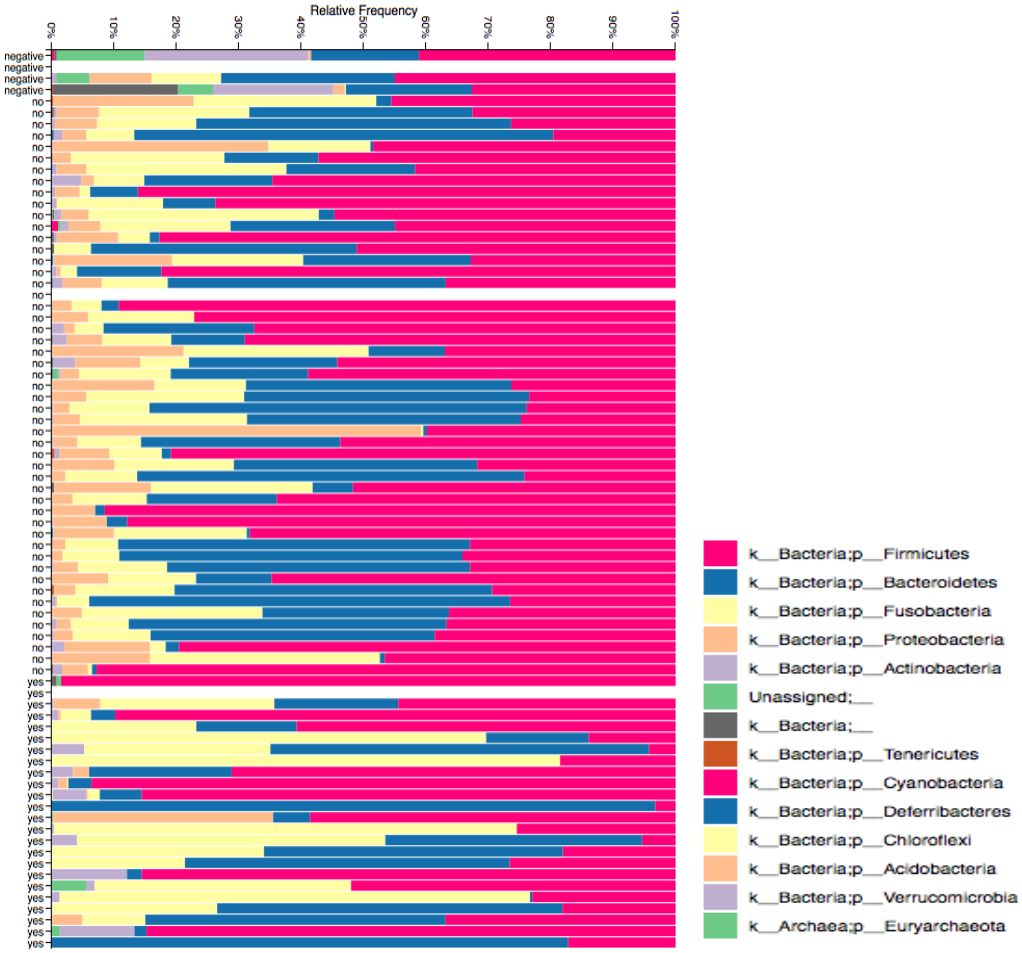


Taxonomic Assignment:

To classify the representative sequences of the set containing dog samples and negative controls, the Greengenes database (last release 13_88) aligned at 99% similarity was chosen. Regarding all the dog samples, there was a clear dominance of the *Firmicutes* and *Bacteroidetes* phyla that were identified in all the samples. The first with abundances varying between 20% and 93% in the shelter group and abundances of 3% to 98% in the household group, and the second with abundances varying between 0.5% and 67% in the shelter group and between 0.4% and 81% in the household group. Other phyla frequently identified were *Fusobacteria*, *Proteobacteria* and *Actinobacteria*.

In the shelter group the phylum *Fusobacteria* was identified in 47/50 of the samples, with abundances varying between 0.6% and 37%, while in the household group it was only identified in 15/23 samples, with values between 2% and 81%. The phylum *Actinobacteria* was also isolated in more samples of the shelter group than in the household group, in the first the abundances varied between 0.1% and 5%, in the second group they were from 0.2% to 12%. On the same note, the phylum *Proteobacteria* was identified in 49/50 samples of the shelter group, with relative frequencies varying between 0.2% and 59%, while in the household group it was only identified in 7/23 samples, with relative frequencies from 0.2% to 35% (Figure 14).

Figure 14 - Bar plot for the taxonomic composition (phylum level) of the dog and negative control samples, grouped by human contact (negative control, no and yes).



The Fisher exact test showed that the only phyla where the frequency was statistical significant different between the shelter and household dogs were the *Fusobacteria* and *Proteobacteria* (Table 4).

Table 4 - Relative abundance and frequency of the most predominant phyla in household and shelter dogs.

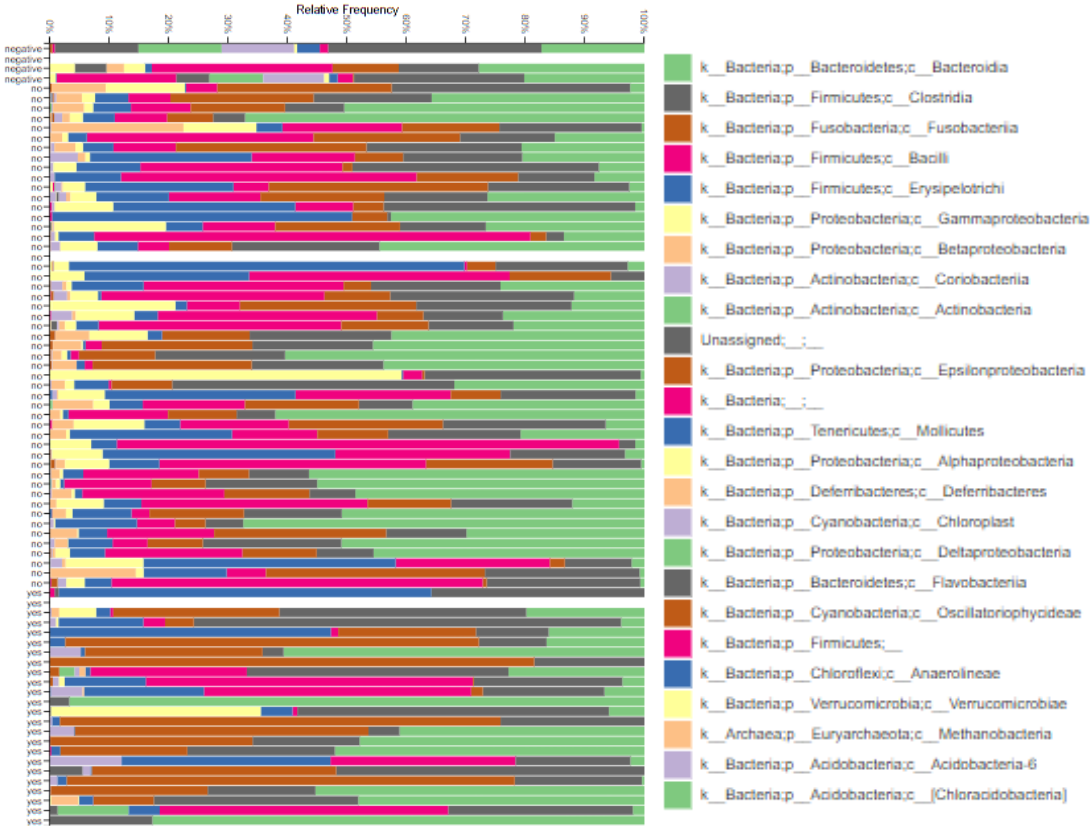
Phylum level	Dogs household N=23		Dogs shelter N=50		p-value
	Relative abundance min.–max. %	n, (%)	Relative abundance min.–max. %	n, (%)	
Firmicutes	3.0-98.0	23, (100)	20.0-93.0	50, (100)	1
Bacteroidetes	0.4-81.0	23, (100)	0.5-67.0	50, (100)	1
Fusobacteria	2.0-81.0	15, (65.2)	0.6-37.0	48, (96.0)	0.003*
Proteobacteria	0.2-35.0	7, (30.4)	0.2-59.0	48, (96.0)	<0.00001*
Actinobacteria	0.2-12.0	12, (52.2)	0.1-5.0	34, (68.0)	0.2054

* Indicates statistical significance. A p value of ≤ 0.05 was considered and the Bonferroni p value adjustment method was used to account for multiple comparisons.

The classes *Bacteroidia*, *Clostridia,* *Fusobacteriia*, *Bacilli*, *Erysipelotrichi* and *Gammaproteobacteria* were the predominant groups (Figure 15). The *Bacteroidia* class was the most predominant one in both the shelter group, with identification in 49/50 animals with relative frequencies varying from 0.6% to 67%, and the household group, where it was identified in 19/23 samples with abundances between 0.4% and 98%. Following was the *Clostridia* class, it was identified in 50/50 of the shelter samples and in 23/23 household samples, in the first group it had abundances varying from 0.6% to 47%, and in the second from 3% to 71%.

Three groups where there was a clear difference between the shelter and household group were the *Bacilli*, *Gammaproteobacteria* and *Fusobacteriia*. The *Baciili* class was identified in 49 samples of the shelter group, with relative abundances going from 0.5% to 84%, however it was only identified in 10/23 household samples, with relative abundances varying between 0.5% and 55%. As for the *Gammaproteobacteria* it was identified in 47/50 of the shelter group samples and only in 5/23 of the household group samples, in the first it had relative frequencies from 0.1% to 59% and in the second from 0.1% to 35%. The *Fusobateria* class was identified in 16/23 of the household samples and in 48/50 of the shelter samples, in the first group the relative abundances varied between 0.2% and 81.5% and in the second group they ranged from 0.4% to 36%

Figure 15 - Bar plot for the taxonomic composition (class level) of the dog and negative control samples, grouped by human contact (negative control, no and yes).



The Fisher exact test showed that the classes where the frequency was statistical significant different between the shelter and household dogs were the *Bacilli*, *Erysipelotrichi* and *Gammaproteobacteria* (Table 5).

Table 5 - Relative abundance and frequency of the most predominant classes in household and shelter dogs.

Class level	Dogs household N=23		Dogs shelter N=50		p-value
	Relative abundance min.–max. %	n, (%)	Relative abundance min.–max. %	n, (%)	
Bacteroidia	0.4-98.0	19, (82.6)	0.6-67.0	49, (98.0)	0.0317
Clostridia	3.0-71.0	23, (100)	0.6-47.0	50, (100)	1
Fusobacteriia	0.2-81.5	16, (69.6)	0.4-36	48, (96.0)	0,0034
Bacilli	0.5-55.0	10, (43.5)	0.5-84.0	49, (98.0)	<0,00001*
Erysipelotrichi	0.1-62.7	18, (78.3)	0.3-66.5	50, (100)	0,0022*
Gammaproteobacteria	0.1-35.0	5, (21.7)	0.1-59.0	47, (94.0)	<0,00001*

* Indicates statistical significance. A p value of ≤ 0.05 was considered and the Bonferroni p value adjustment method was used to account for multiple comparisons.

At the genus level, a various genera were identified, being that the dominant ones were the *Bacteroides*, *Prevotella*, *Lactobacillus* and *Clostridium.* There is a non-identified genus, belonging to the *Fusobacteriales* order that was also frequently identified, especially in the household group (Figure 16).

The *Bacteroides* genus was identified in 44/50 of the shelter samples and in 15/23 samples of the household group, in the first it has relative abundances ranging from 0.1% to 23%, while in the second it had values from 0.4% to 97%. The *Prevotella* genus was identified in 37/50 samples from the shelter group with relative frequencies from 0.2% to 55%, and in 13/23 samples from the household group with relative abundances from 0.3% to 69%. The *Clostridium* genus was identified in 47/50 samples of the shelter group samples and in 20/23 samples of the household group, in the first group the abundance varied between 0.1% and 26% and in the second group varied between 0.2% and 53%.

As for the *Lactobacillus* genus it was only identified in the shelter group, in 41/50 samples, and in relative frequencies varying from 0.5% to 73%. A non-identified genus belonging to the *Enterobacteriaceae* family was also identified in 39/50 samples of the samples from the shelter group, but only in 3/23 samples from the household group, in the first the relative abundances went from 0.2% to 59% and in the second one the samples had a relative frequency between 0.2% and 35%. The *Streptococcus* genus was identified in 38/50 of the shelter samples, with a relative abundance ranging from 0.1% to 57%, and in 5/23 samples from the household group, with a minimum relative abundance of 0.05% and a maximum of 26%.

Figure 16 - Bar plot for the taxonomic composition (genus level) of the dog and negative control samples, grouped by human contact (negative control, no and yes).

The Fisher exact test showed that the classes where the frequency was statistical significant different between the shelter and household dogs were the *Bacilli*, *Erysipelotrichi* and *Gammaproteobacteria* (Table 6).

Table 6 - Relative abundance and frequency of the most predominant classes in household and shelter dogs.

Genus level	**Dogs household N=23**		**Dogs shelter N=50**		**p-value**
	Relative abundance min.–max. %	**n, (%)**	**Relative abundance min.–max. %**	**n, (%)**	
Bacteroides	0.4-97.0	15, (65.2)	0.1-23.0	44, (88.0)	0.0291
Prevotella	0.3-69.0	13, (56.5)	0.2-55.0	37, (74.0)	0.1771
Lactobacillus	0.0	0, (0.0)	0.5-73.0	41, (82.0)	< 0.00001*
Clostridium	0.2-53.0	20, (86.9)	0.1-26.0	47, (94.0)	0.3715
Enterobacteriaceae (family)	0.2-35.0	3, (13.0)	0.2-59.0	39, (78.0)	< 0.00001*
Streptococcus	0.05-26.0	5, (21.7)	0.1-57.0	38, (76.0)	0.001*

* Indicates statistical significance. A p value of ≤ 0.05 was considered and the Bonferroni p value adjustment method was used to account for multiple comparisons.

Comparing the microbial composition of two or more populations based on the OTUs in the sample is not the same as compare the abundance of the taxa in the microbial ecosystems from which the sample is obtained. In this study was performed Analysis of composition of microbiomes (ANCOM) test to understand if there were statistical significances between the microbial composition between groups. This method compares abundances of genera to determine if they change significantly between populations or environments (Mandal et al., 2015). The higher the W value the more significant the differences in abundance levels between groups.

Table 7 ANCOM statistically significant features for the phyla differently observed in the dog and negative control samples with and without human contact.

Phylum	Rejected null hypothesis	W
Unassigned;__	True**	13
k__Bacteria;p__Firmicutes	True**	10
k__Bacteria;p__Fusobacteria	True**	11
k__Bacteria;p__Proteobacteria	True**	13

** Indicates statistical significance; OTU- Operational taxonomic unit

Table 8 ANCOM statistically significant features for the classes differently observed in the dog and negative control samples with and without human contact.

Class	Rejected null hypothesis	W
Unassigned;__;__	True**	23
k__Bacteria;p__Actinobacteria;c__Actinobacteria	True**	18
k__Bacteria;p__Firmicutes;c__Bacilli	True**	22
k__Bacteria;p__Firmicutes;c__Erysipelotrichi	True**	20
k__Bacteria;p__Fusobacteria;c__Fusobacteriia	True**	17
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	True**	19
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	True**	22

** Indicates statistical significance; OTU- Operational taxonomic unit

Table 9 ANCOM statistically significant features for the genera differently observed in the dog and negative control samples with and without human contact.

Genera	Rejected null hypothesis	W
Unassigned;__;__;__;__;__	True**	126
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae;g__Bifidobacterium	True**	115
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;__	True**	123
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__	True**	117
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Geobacillus	True**	112
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	True**	129
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Acidaminococcus	True**	122
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Megasphaera	True**	120

** Indicates statistical significance; OTU- Operational taxonomic unit

The ANCOM analysis of the dog samples showed that there was a statistical significant difference of the relative abundances between the shelter and household group for the *Firmicutes*, *Fusobacteria* and *Proteobacteria* phyla (Table 7); *Actinobacteria, Bacilli, Erysipelotrichi, Fusobacteria, Betaproteobacteria* and *Gammaproteobacteria* classes (Table 8); *Bifidobacterium, Geobacillus, Lactobacillus, Acidaminococcus* and *Megasphaera* genera (Table 9).

3.3.3 Analysis of the Cat Samples

The quality-controlled feature table obtained with DADA2 was filtered to include only the 22 samples belonging to “Cat” and the 4 negative controls (Figure 17, Figure 18). Besides the “negative_control_4” sample that has 0 sequence counts, the minimum sequence count is 1 302, belonging to the “negative_control_5” sample. The sample with highest sequence count is C44, with 38 380 sequences.

Figure 17 - Sample distribution per species.

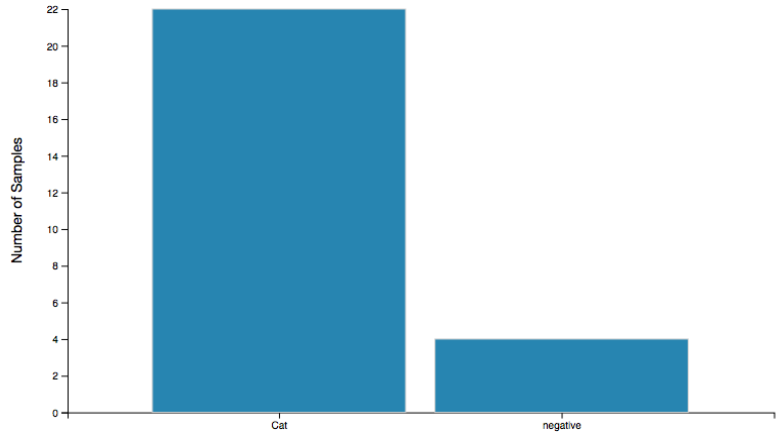
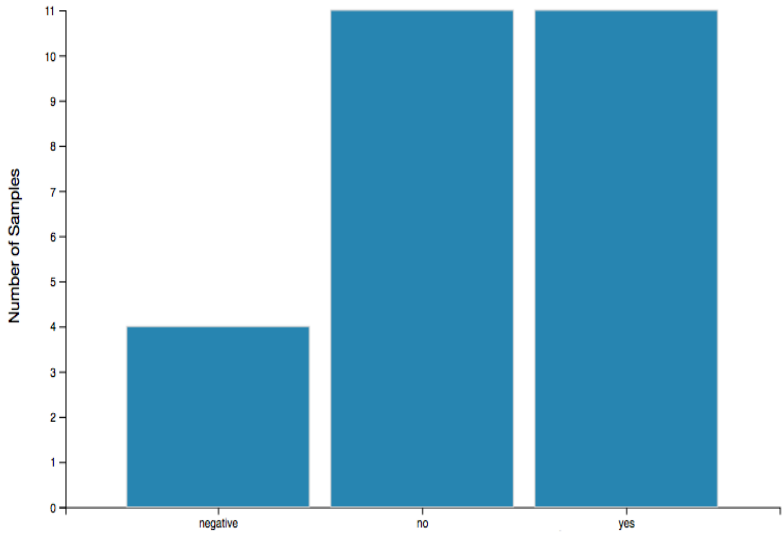


Figure 18 - Sample distribution per human contact.

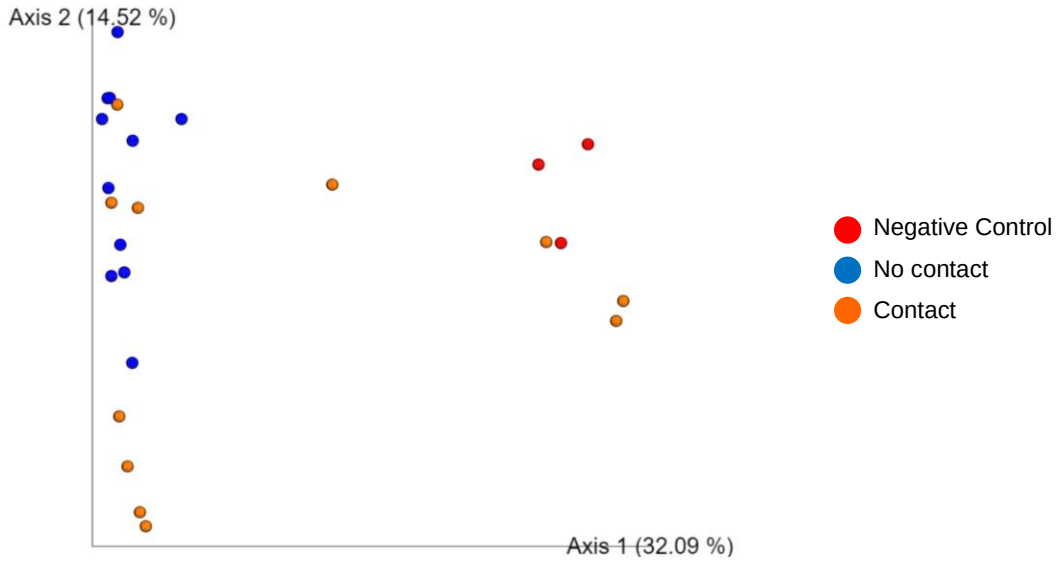


Alpha and Beta Diversity:

The samples were rarefied to **1 302** read depth in according to the sample with the lowest read depth ("negative_control_5").

The PCoA plot was obtained by visualizing the graph obtained in qiime2 (Figure 19). The cats with and without human contact seem to have no separation with the exception of the positive samples for human contact that are sitting with the negative controls.

Figure 19 - PCoA plot for the cat and negative control samples coloured by human contact (yes, no and negative controls).



Alpha Diversity:

The Kruskal-Wallis nonparametric test was applied to obtain the alpha diversity of the 3 groups (with human contact, without human contact, and negative control group) and the observed OTUs were statistical significant different between the groups (p-value <0.00001) (Figure 20, Table 10). It is also possible to observe that the groups without human contact had more observed OTUs than the group with human contact.

Figure 20 - Alpha diversity boxplots for the cat and negative control samples according to human contact (yes, no and negative control).

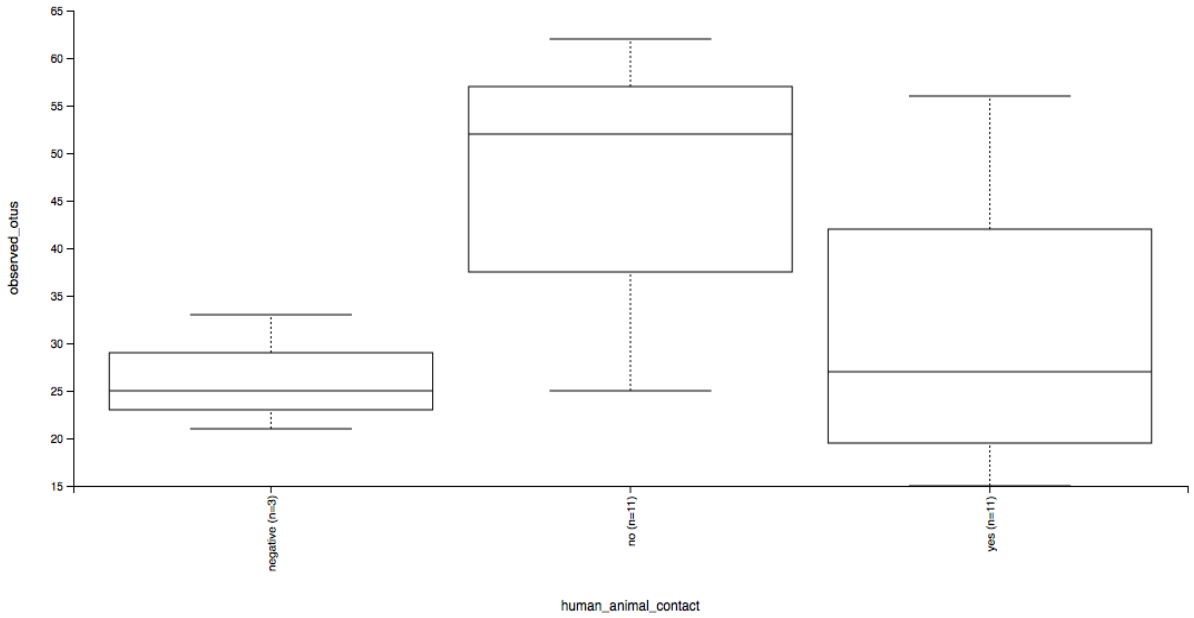


Table 10 Pairwise Kruskal-Wallis test for the cat and negative control samples according to human contact (yes, no and negative control).

		H	p-value	q-value
Group 1	**Group 2**			
negative (n=3)	no (n=11)	4.447506	0.034952	0.052428
	yes (n=11)	0.054545	0.815335	0.815335
no (n=11)	yes (n=11)	5.594532	0.018017	0.052428

The alpha diversity, i.e. taxonomic diversity within a sample, for all groups obtained a p-value of < 0.05, which means that there is statistical significant difference in the organismal richness of the sample and the evenness of organisms' abundance distribution between groups. If the two groups, household and shelter, are compared pairwise, a p-value <0.05 is obtained. This value shows that there is a statistical significant difference between the groups in what alpha diversity is concerned.

Beta Diversity:

Beta diversity analysis was used to determine the faecal microbiome composition diversity between the animals using the Principal coordinate analysis (PCoA) plot based on the unweighted – unifrac distance. The PERMANOVA test was used to test differences in mean community structure within the 3 groups (with human contact, without human contact, and negative control group) and it was observed statistical significant difference (p-value =0.001) (Figure 21, Figure 22, Figure 23, Table 11).

Table 11 – PERMANOVA test results for the cat samples

Sample size	25
Number of groups	3
Test statistic	3.53867
p-value	**0.001**
number of permutations	999

Figure 21 - Box plot of the distance the negative control, cats without human contact and cats with human contact to the negative control.

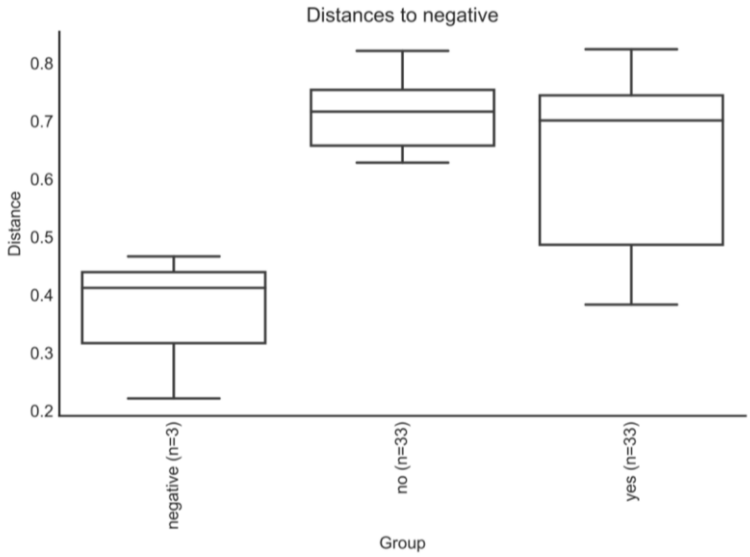


Figure 22 - Box plot of the distance the negative control, cats without human contact and cats with human contact to cats without human contact.

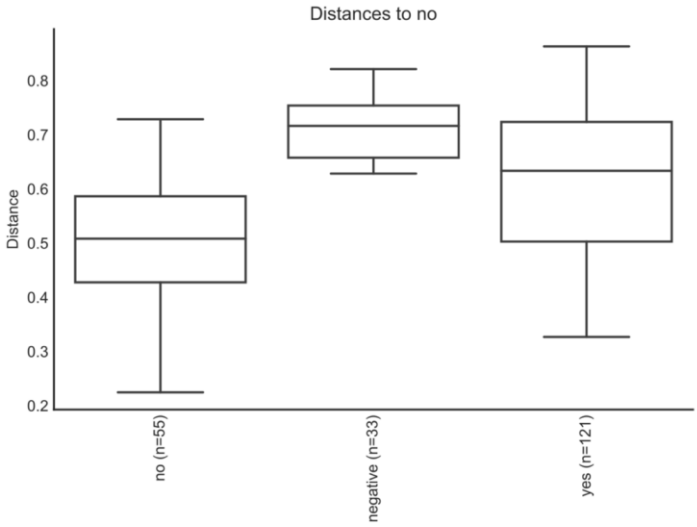
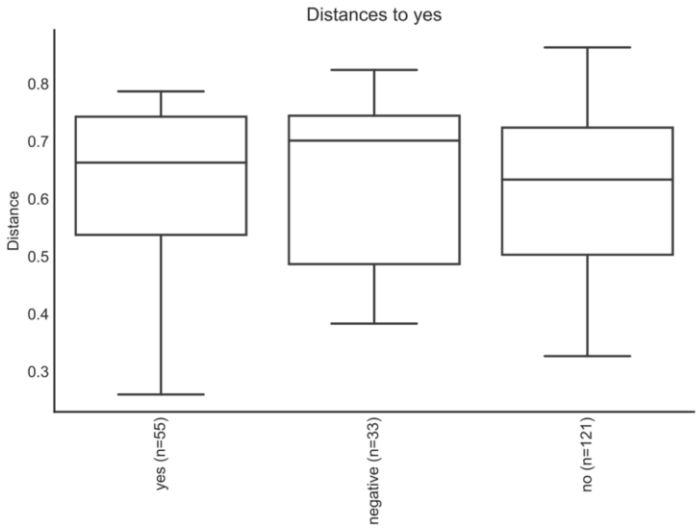


Figure 23 - Box plot of the distance the negative control, cats without human contact and cats with human contact to cats with human contact.

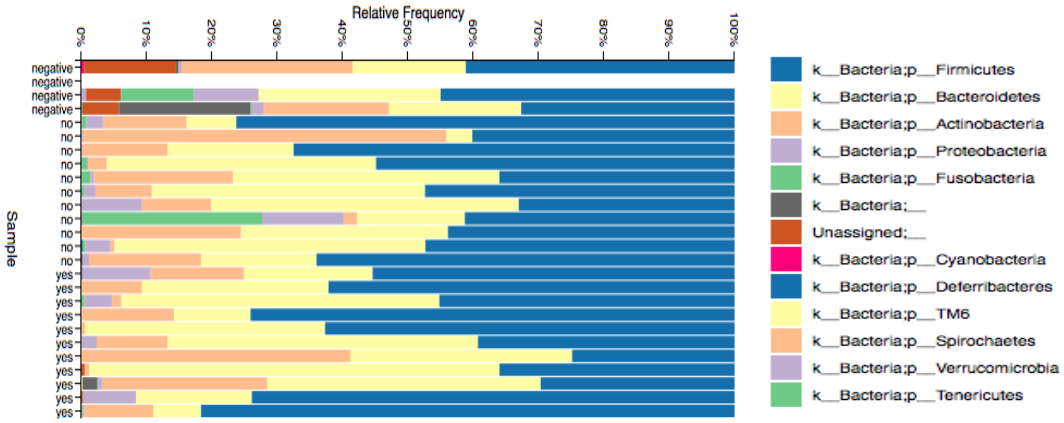


Taxonomic Assignment:

As classifier, the Greengenes database (last release 13_88) aligned at 99% similarity was chosen.

Regarding the cat samples, from both household and shelter groups, there was a clear dominance of the *Firmicutes* in both the household group, with abundances varying between 24.9% and 81.7%, and the shelter group, with abundances varying between 33% and 76.3%. The *Bacteroidetes* phyla was identified in all the samples, with abundances varying between 7.3% and 62.8% in the household group and between 4% and 47.6% in the shelter group. The other phyla that was also identified in all the household and shelter samples was the *Actinobacteria,* with relative abundances varying from 0.6% to 41.2% in the first one, and 0.7% to 55.6% in the shelter group (Figure 23, Table 10). The *Proteobacteria* phyla was identified in 6/11 samples from the household group and in 10/11 samples from the shelter group, the relative abundances were quite similar for both groups and varied between 0.1% and 12%. It is interesting to note that the phylum *Fusobacteria* was only identified in three samples from the household group, with a relative abundance varying between 0.1% and 0,4%, and in 6/11 samples from the shelter group, with relative abundances varying between 0.4% and 28%.

Figure 24 - Bar plot for the taxonomic composition (phylum level) of the cat and negative control samples, grouped by human contact (negative control, no and yes).



The Fisher exact test showed that the frequency was not statistical significant different between the shelter and household cats in what the phylum level was concerned (Table 12).

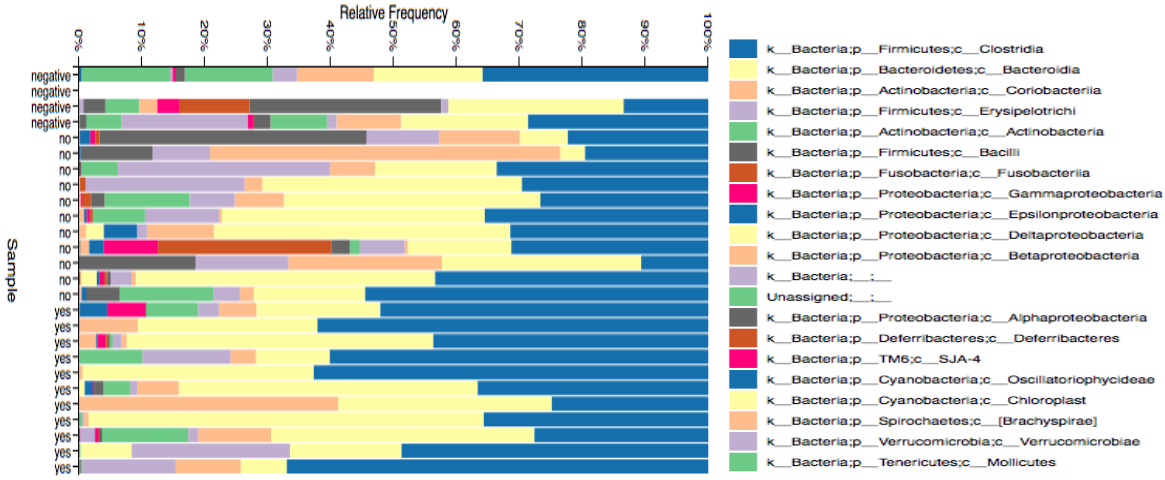
Table 12 - Relative abundance and frequency of the most predominant phyla in household and shelter cats.

Phylum level	Cats household N=11		Cats shelter N=11		p-value
	Relative abundance min.–max. %	n, (%)	Relative abundance min.–max. %	n, (%)	
Firmicutes	24.9-81.7	11, (100)	33.0-76.3	11, (100)	1
Bacteroidetes	7.3-62.8	11, (100)	4.0-47.6	11, (100)	1
Actinobacteria	0.6-41.2	10, (90.9)	0.7-55.6	11, (100)	0.5
Proteobacteria	0.2-10.5	6, (54.5)	0.1-12.5	10, (90.9)	0.1486
Fusobacteria	0.1-0.4	3, (27.3)	0.4-27.6	6, (54.5)	0.1933

The classes *Clostridia* and *Bacteroidia* were isolated in higher percentage than any other class in both groups and were identified in all the samples. The *Clostridia* class had relative abundances varying between 25% and 67% in the household group and between 11% and 25% in the shelter group. The *Bacteroidia* class had abundances from 7% to 63% in the household group and from 4% to 48% in the shelter group (Figure 24, Table 11). Following were the *Coriobacteriia*, *Erysipelotrichi* and *Actinobacteria* classes, with about the same number of samples and relative abundances in both groups.

It is important to emphasize two classes that were quite different when comparing both groups, the *Bacilli* class and the *Fusobacteria* class. The first class was observed in 4/11 of the household samples, with relative frequencies between 0.2% and 2%, and in 9/11 of the shelter samples, with relative frequencies between 0.1% and 42%. The second class was also observed in 3/11 of the household samples, with abundances between 0.1% and 0.4%, and in 6/11 of the shelter samples, with abundances varying from 0.4% to 28%.

Figure 25 - Bar plot for the taxonomic composition (class level) of the cat and negative control samples, grouped by human contact (negative control, no and yes).



The Fisher exact test showed that there wasn't any statistical significant difference between the household and shelter cats in the frequency of any class (Table 13).

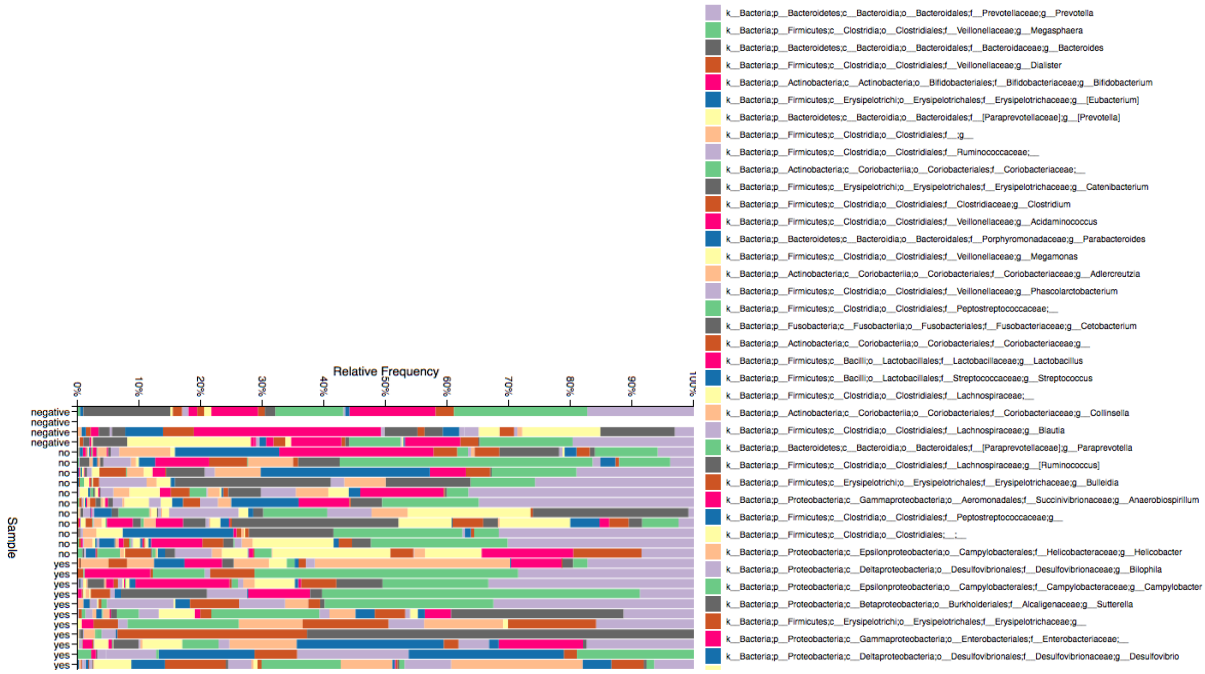
Table 13 - Relative abundance and frequency of the most predominant classes in household and shelter cats.

Class level	Cats household N=11		Cats shelter N=11		p-value
	Relative abundance min.–max. %	n, (%)	Relative abundance min.–max. %	n, (%)	
Clostridia	24.9-67.0	11, (100)	10.7-54.5	11, (100)	1
Bacteroidia	7.3-62.8	11, (100)	4.0-47.6	11, (100)	1
Coriobacteriia	0.6-41.2	10, (90.9)	0.4-55.6	11, (100)	1
Erysipelotrichi	0.3-25.2	8, (72.2)	1.6-33.7	11, (100)	0.2143
Actinobacteria	0.2-13.7	6, (54.5)	1.6-14.9	5, (45.5)	0.669815
Bacilli	0.2-1.5	4, (27.3)	0.1-42.4	9, (81.9)	.030148
Fusobacteria	0.1-0.4	3, (27.3)	0.4-27.6	6, (54.5)	0.193297

At the genus level, several genera were identified, being that the dominant ones were the *Prevotella*, *Megasphaera* and *Bacteroides.* The *Prevotella* genera was identified in all of the shelter samples, with relative frequencies varying between 0.8% and 37%, and in 9/11 of the household samples, with relative frequencies varying from 6% to 33%. The *Megasphaera* genera was identified in 9/11 of the shelter samples, with abundances from 3% to 22%, and in 7/11 of the household samples, with abundances from 1% to 52%. As for the *Bacteroides* genera it was observed in 8/11 of the household samples, with abundances varying between 0.5% and 63%, and in 9/11 of the shelter samples, with abundances going from 0.2% to 25%. In some of the samples the genus *Dialister, Bifidobacterium* and *Clostridium* were also identified (Figure 25, Table 12). The *Dialister* was observed in 6/11 of the household samples,

with relative frequencies varying from 2% to 31%, and in 10/11 samples from the shelter group, with relative abundances varying from 0.2% to 11%. As for the *Bifidobacterium* it was observed in and equal number of samples from each group, 5/11 samples each, the abundances were also similar, going from 0.5% to 14%. The *Clostridium* genera was also observed in the same number for both groups, 9/11 samples each, with relative abundances going from 0.3% to 14% in the household group, and 0.2% to 5% in the shelter group.

Figure 26 - Bar plot for the taxonomic composition (genus level) of the cat and negative control samples, grouped by human contact (negative control, no and yes).



As for the phylum and class levels, for the genus level the Fisher exact test showed that there wasn't any statistical significant difference between the household and shelter cats (Table 14).

Table 14 - Relative abundance and frequency of the most predominant genera in household and shelter cats.

Genus level	Cats household N=11		Cats shelter N=11		p-value
	Relative abundance min.–max. %	n, (%)	Relative abundance min.–max. %	n, (%)	
Prevotella	24.9-67.0	9, (81.9)	10.7-54.5	11, (100)	1
Clostridium	7.3-62.8	9, (81.9)	4.0-47.6	8, (72.7)	1
Megasphaera	0.6-41.2	7, (63.6)	0.4-55.6	9, (81.9)	0.6351
Bacteroides	0.3-25.2	8, (72.7)	1.6-33.7	9, (81.9)	1
Dialister	0.2-13.7	6, (54.5)	1.6-14.9	10, (90.9)	0.1486
Bifidobacterium	0.1-0.4	5, (45.5)	0.4-27.6	5, (45.5)	1

To explore if the differences observed in the plots for cats with and without human contact are statistically significant, the ANCOM (Analysis of Composition of Microbiomes) was used.

Table 15 ANCOM statistically significant features for the phyla differently observed in the cat and negative control samples with and without human contact.

Phylum	Rejected null hypothesis	W
Unassigned;_	True**	11

** Indicates statistical significance; OTU- Operational taxonomic unit

Table 16 ANCOM statistically significant features for the classes differently observed in the cat and negative control samples with and without human contact.

Class	Rejected null hypothesis	W
Unassigned;__;__	True**	15
k__Bacteria;p__Bacteroidetes;c__Bacteroidia	True**	11
k__Bacteria;p__Firmicutes;c__Clostridia	True**	11

** Indicates statistical significance; OTU- Operational taxonomic unit

The ANCOM analysis of the cat samples showed that there wasn't a statistical significant difference of the relative abundances between the shelter and household group for any specific phyla (Table 15). However, there was a statistical significant difference for *Bacteroidia* and *Clostridia* classes (Table 16). There was not a statistical significant difference between the two groups on the genus level.

3.4 Discussion

To our knowledge this is the first study that compares the microbiome of household pets to those of shelter dogs and cats. The main objectives were to describe the normal microbiome of healthy animals, as well as to observe if there was a significant difference in the microbiome of animals presently living in different environments (household *vs* shelter).

In this study, for analyzing the gut microbiome from dogs and cats the Illumina MiSeq platform was used. The metagenomic sequencing technique has several advantages and disadvantages. Comparing to culture methods, it is possible to have results faster and with greater accuracy, since it allows the identification of unculturable bacteria. However, it is also more sensitive to any kind of contamination. Among the several next generation sequencing systems, this platform is able not only able to generate long and high-quality sequence reads, but also to have the lowest error rates; in addition, it is the most cost-effective platform, being the most suitable for small investigations like this one. (Caporaso et al., 2012; Motro & Moran-Gilad, 2017; Quail et al., 2012; Salipante et al., 2014)

As already described in previous studies the main component of the dog and cat microbiome is the bacteria domain (Moon et al., 2018). In this study we got values of about 99% for this domain. Comparing the dog and cat samples we can see they are well separated, which means

there is a clear difference between the dog and cat's microbiome, confirming what was already described in a previous study (Handl et al., 2011).

It is important to note that the negative controls (n=3) lie in the middle of the samples. A recent study indicates that commercial DNA extraction kits are usually contaminated, and that contamination may vary between brands and even between batches of the same kit (D. Kim et al., 2017), this may justify the fact that our samples had some level of bacterial identification. Interestingly, most of the articles regarding the gut microbiome do not mention the execution of parallel negative controls during the DNA extraction step. It is possible that they are not routinely performed or if it is done, authors may have omitted the results. This fact makes it harder to assess the results obtained regarding the negative controls.

Canine gut microbiome

Regarding only the dog samples it is possible to observe that dogs with human contact (household) and the dogs without human contact (shelter) have different gut microbiome results. These findings, corroborate with the suspicion that the environment where animals live may influence the gut microbiome composition (Cho & Blaser, 2012; Kurokawa et al., 2007).

The alpha diversity, i.e. taxonomic diversity within a sample, for all groups indicated that there was a statistical significant difference in the organismal richness of the sample and the evenness of organisms' abundance distribution. It is also possible to observe that the group without human contact has a more diverse population, whereas the group with human contact has a less diverse population. This fact is of extreme importance because, in studies regarding human health, it has been shown that a serious number of diseases have been associated with lower microbiome diversity (Minamoto et al., 2015; Morgan & Huttenhower, 2012). This decrease in the diversity of the gut microbiome may have many factors, including obesity, higher antimicrobial intake (Ianiro et al., 2016) or the administration of proton pump inhibitors, like omeprazole (Garcia-Mazcorro et al., 2012). However, some other studies in dogs have not found an association between obesity and the gut microbiome diversity (Handl et al., 2013). Some more studies are needed to assess which factors may have an influence in the decrease of the gut microbiome taxonomic diversity.

As for the beta diversity, i.e. taxonomic diversity between samples, it was obtained a p-value of 0.001, meaning that there is a statistical significant difference in organismal composition between the samples. Again, the hypothesis that the environment may have a strong influence in the gut microbiome is reinforced. Although some studies would be needed to prove this, one other hypothesis we might consider is that human influence is much stronger in the household group.

Going further into the analysis, it is important to compare the taxonomic groups of this study with some of the other existing studies. The results obtained in the present study were quite similar to the ones obtained in previous reports using the Illumina platform to describe the gut microbiome of the dog (Coelho et al., 2018; Herstad et al., 2017; Igarashi et al., 2014; Kim et

al., 2017; Li et al., 2017). As previously described, the major bacterial phyla identified in the dog samples from both groups were *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, *Proteobacteria* and *Actinobacteria*. There was a statistical significant difference between the shelter group and the household group for the *Fusobacteria* phylum ($p=0.003$) and the *Proteobacteria* phylum ($p<0.00001$) which were in higher proportion in the shelter group. The *Proteobacteria* phylum, regularly identified in the faecal microbiota of healthy dogs, is the most diverse bacterial phylum composed by gram-negative bacteria, including several opportunistic pathogens like *E. coli*, *Salmonella*, *Campylobacter*, and many other (Moon et al., 2018). The *Proteobacteria* are one of the earlier colonizers of the gut, and contribute to a higher functional variation and maintenance of a balanced gut microbial community (Bradley & Pollard, 2017; Shin, Whon, & Bae, 2015). It is suggested that higher numbers of *Proteobacteria* in the gut microbiome is associated with dysbiosis, as well as local and systemic inflammation or metabolic disorders, such as obesity or diabetes (Bradley & Pollard, 2017; Shin et al., 2015).

In previous studies about faecal microbiome of healthy dogs, the relative abundances of *Proteobacteria* ha values ranging from 0% to 22% (Garcia-Mazcorro et al., 2012; Hand et al., 2013). In our study the household dogs had relative abundances quite similar to these studies, ranging from 0.2% to 35%. However, in the shelter group these values ranged between 0.2% and 59% which is rather higher that the values already described. This difference may be justified by the use of different techniques for the 16S sequencing, by the different environments these animals were raised and born or by the diet administered. In fact, a recent study has shown that animals eating raw meat had an higher percentage of *Proteobacteria* than the ones eating commercial diet, since some of the shelter animals are rescued in the streets and we have no information of the food they have eaten until they go to the shelter it is a possibility we have to consider, however more background information and more studies would be needed for reaching a conclusion (Sandri, Dal Monego, Conte, Sgorlon, & Stefanon, 2017).

Regarding the class level, the more representative classes were *Bacteroidia*, which belongs to the *Bacteroidetes* phylum, the *Clostridia*, *Bacilli* and *Erysipelotrichi*, all belonging to the *Firmicutes* phylum, class *Fusobacteria*, from the *Fusobacteria* phylum, and the *Gammaproteobacteria* class, which belongs to the *Proteobacteria* phylum. The only two classes where there was a statistical significant difference between the two groups were the *Bacilli* and *Gammaproteobacteria*. It is known that in the gastrointestinal environment the *Gammaproteobacteria* is the most representative class from the *Proteobacteria* phylum and part of the normal faecal microbiota in dogs (Moon et al., 2018).

Comparing these results to those from a previous study, even though the most prevalent classes were the same, it is possible to see some differences in the range of relative abundances of the various classes (Igarashi et al., 2014). While in the study from Igarashi (2014) the *Bacteroidia* class relative abundances ranged from 0.36% to 9.82%, in this study

the maximum value was higher in both the household (98%) and the shelter (67%) group. The other class where there was a difference between the two studies was the Bacilli class, with a maximum relative abundance of 11.90%, while in our study the relative abundances had a maximum of 84% in the shelter group and of 55% in the household group.

These differences are hard to explain since that, to our knowledge, there are no studies that give an explanation about the increased frequency of the *Bacteriodia* class in the gut microbiome of dogs. One explanation may be the fact that the geographic areas of these studies are very different (Japan *versus* Portugal), and a study shows that social structure can have an important influence in the vertical transmission and the flow of microbes and microbial genes among members of a household, be it for the contact the animals have with the other house members, the diet or many other factors (Yatsunenko et al., 2012). There is also the possibility that, like in the human microbiome, more than one enterotype of the gut microbiome exist for the dog species, meaning that there can be more than one host–microbial symbiotic state and that they might respond differently to diet and drug intake (Arumugam et al., 2011). One other hypothesis we must consider is the fact that despite the sequencing method is the same for both studies, the DNA extraction method was different.

As for the genus level, it is important to note that there was some heterogenicity between samples, being that the most prevalent genera in the majority of the samples were *Bacteroides*, *Prevotella*, *Lactobacillus*, *Clostridium* and *Streptococcus.* The only genera where there was a statistical significant difference between the number of identifications in the shelter group and the household group were the one belonging to the *Enterobacteriaceae* family, which was identified in higher number in the shelter animals, and the *Lactobacillus*, this genus wasn't identified in any of the household samples.

The higher number of samples with the identification of the *Enterobacteriaceae* family in our samples may be due to dysbiosis or the inclusion of raw meat in the animals diet (Sandri et al., 2017), however we don't have enough data about all the samples, especially the ones belonging to the shelter group, therefore it is difficult to make an absolute conclusion. As for the *Lactobacillus*, since to the best of our knowledge there are no studies concerning this genus and its role in the gut microbiome of the dog, we could not find a justification for the existence of a higher number of samples in the shelter group containing these bacteria.

However, comparing with the study from Igarashi (2014), in which the relative abundance of *Lactobacillus* ranged between 0.01% and 5.02%, we can see that in the shelter group from our study, this range was much higher, with the values varying between 0.5% and 73%. In the same study, the *Enterobacteriaceae* family had a relative abundance varying between 0% and 3.42%, in our study it varied between 0.2% and 59% for the shelter group and between 0.2% and 35% for the household one. This differences in the range of the relative abundances may be justified by the different geographic areas where the studies were conducted (Yatsunenko et al., 2012).

For the other genera, there were no statistical significant difference between the number of samples in the shelter group and the household group. However, comparing with another existing study, there was a difference in the relative abundances of the genera *Streptococcus*, *Prevotella*, *Bacteroides* and *Clostridium*. In Igarashi (2014) the *Streptococcus* genus had relative abundances ranging from 0% to 0.13%, in our study it ranged between 0.1% and 57% for the shelter group, and between 0.05% and 26% for the household group; as for the *Prevotella* genus the maximum value obtained in our study was quite higher, 55% for the shelter group and 69% for the household group, than the one obtained in the previous study, 6.91%. The *Bacteroides* genus case is a bit different since the relative abundances were also very different among the two groups from our study, in the shelter group it ranged between 0.1% and 23%, while in the household group it went up to 93%, in the Igarashi (2014) study it ranged from 0.07% to 6.89%. The *Clostridium* relative abundances varied between 2.42% and 51.27% in the Igarashi (2014) study, the values from the household group were very similar to these ones, going from 0.2% to 53%, however in the shelter group these values were substantially lower, ranging only between 0.1% and 26%. It is known that the *Clostridiaceae* family abundance tends to be higher in animals fed with natural diets, consequently we could make the assumption that the same is true for the *Clostridium* genus (Kim et al., 2017). Unfortunately, this theory doesn't apply to our study since all the animals were being fed with commercial diet at the time of the sample collection. What we might consider a possibility is the fact that all the commercial dry foods have different percentages of protein, carbohydrates and lipids, more studies would be needed to study these differences. Another aspect that we have to consider is the variety of breeds that exist for the canine species and that can possibly have an influence in the gut microbiome.

The ANCOM analysis of the dog samples showed that there was a statistical significant difference of the relative abundances between the shelter and household group for the *Firmicutes*, *Fusobacteria* and *Proteobacteria* phyla*; actinobacteria*, *Bacilli*, *Erysipelotrichi*, *Fusobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* classes; *Bifidobacterium*, *Lactobacillus*, *Acidaminococcus* and *Megasphaera* genera.

Previous studies have shown that shelter dogs have a higher risk of carrying resistant bacteria, such as plasmidic AmpC β-lactamase producing *Escherichia coli*, which indicates that in terms of carriage of resistant bacteria the shelter dogs are worse than the household dogs (Belas, Salazar, Gama, Couto, & Pomba, 2014). However, in terms of microbiome composition the shelter dogs seem to have a less predisposition to develop disease, meaning that the shelter dogs are subjected to a less negative modulation of the gut microbiome than the household dogs. Hence, we need to think what is being done wrong in the household dogs and not the shelter dogs.

Feline gut microbiome

Concerning the cat samples, the distribution regarding the number of samples is the same for both groups. Observing the PCoA plot (Figure 19) it is not clear that the samples from the household groups are well separated from the shelter samples, meaning that meaning that it can't be assumed that the environment the animals reside in and the amount of contact they have with humans may have an influence in their gut microbiome.

Analyzing the alpha diversity of the groups it is possible to state that there is a clear difference between both groups (p=0.023) and that the household group has a lower number of observed OTUs than the shelter group. This lower number of OTUs means that the diversity is higher in the shelter group, and as in stated for the dog samples, it has been shown that some diseases have been associated with lower microbiome diversity (Minamoto et al., 2015; Morgan & Huttenhower, 2012). The p value obtained for the beta diversity was of 0.001, meaning that there is a difference between the two groups in what the organismal composition is concerned. This difference may be justified by the influence of the environment the animals live and/or by the fact that household animals are subjected to different influences from the shelter animals, such as contact with humans, antimicrobials intake, diet, among others.

The predominant phyla in our study were *Firmicutes* and *Bacteroidetes,* being that they were identified in all the samples and with high relative abundances, followed by *Actinobacteria*, *Proteobacteria* and *Fusobacteria*, this results are consistent with previous studies in healthy cats (Duarte et al., 2016; Minamoto, Hooda, Swanson, & Suchodolski, 2012; Ritchie et al., 2010; Tun et al., 2012). In contrast with the dog samples in the cat analysis we could not find any statistical significant difference between the shelter and household groups in what the phylum identification is concerned.

At the class level, the most predominant ones were *Clostridia*, *Bacteroidia*, *Coriobacteriia* and *Erysipelotrichi,* other classes that were identified, but in a lower number of samples were *Actinobacteria*, *Bacilli* and *Fusobacteria* which is in accordance with the already existing studies in healthy cats (Duarte et al., 2016; Minamoto et al., 2012; Ritchie et al., 2010). There was also no significant statistical difference between the two groups in the number of isolates containing each class.

It is important to emphasize the *Bacilli* class which had a relative abundance ranging from 0.1% to 42% in the shelter group and from 0.2% to 2% in the household group. Higher relative abundances of this class have been associated with diarrhea in cats (Suchodolski et al., 2015). Even though we had no information about the cats being with alterations of the faeces consistency it is a hypothesis to consider. However more studies would be needed to reach a conclusion. Even though in our study the relative abundance were around the same values for both groups, it is interesting to mention that in the same study the class *Erysipelotrichi* had increased relative abundance in cats with chronic diarrhea (Suchodolski et al., 2015),

Another class that is interesting to refer is the *Coriobacteriia*, which increase has been associated with helminth infections (*Toxocara cati*) in the cat, however, in our study, we had no difference between the shelter and household group concerning this class (Duarte et al., 2016).

The genera more prevalent for both groups were *Prevotella*, *Clostridium*, *Megasphera* and *Bacteroides*, followed by *Dialister* and *Bifidobacterium*. The *Prevotella* and *Megasphera* are usually identified with higher relative abundances in animals fed with dry diets, which is in accordance with our study, however we had lower relative abundance of the *Lactobacillus* genus and higher relative abundance of the *Clostridium* genus than it was expected (Bermingham et al., 2013). These differences may be justified by the fact that in Portugal some animals are mainly fed with dry food but also occasionally with wet food, or even that some cats are not exclusively indoor, meaning that the owners don't know everything they eat. Another aspect that can be considered is the quantity of protein present in the available commercial dry foods, that is known to have an influence in the microbiome of cats (Lubbs, Vester, Fastinger, & Swanson, 2009). Another fact that is important to infer is that in Bermingham (2013) the DNA extraction and sequencing methods were different from the ones used in our study and this may have an influence in the results obtained.

The ANCOM analysis of the cat samples showed that there was only a statistical significant difference of the relative abundances between the shelter and household group for the *Bacteroidia* and *Clostridia* classes.

3.5 Conclusions

To our knowledge, this is the first study that compares the gut microbiome composition between household and shelter companion animals.

With this study we were able to show that canine gastrointestinal microbiome is different from the feline one. However more studies are needed to understand which factors may influence this difference between the two species.

This study also shows that the household dogs and the shelter dogs have different gut microbiomes, demonstrating that the environment where the animals are presently living, as well as the amount of contact they have with humans, may have a great influence in their gut microbiome. Also in this field, more studies are needed in order to understand what other factors may be implied in this difference.

As for the cat groups we couldn't reach the same conclusion. This may be due to the smaller sample size. Therefore, a study including more cats from both groups would be interesting to reach some other conclusions.

Overall, the data obtained in this study may add an important value to the field of veterinary microbiome research contributing to the development of new studies focusing on intrinsic or extrinsic factors that may change it, such as antimicrobials intake, disease states or other.

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